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13. Abstract (Maximum 200 Words) <i>(abstract should contain no proprietary or confidential information)</i> The purpose of this proposal is to isolate and identify estrogen-responsive genes in human breast cancer cells using the chromatin immunoprecipitation (ChIP) protocol. Estrogen receptor (ER)-bound DNA has been isolated using ChIP from human breast cancer MCF-7 (T5) cells (ER positive and hormone dependent). Southern blotting analysis shows that ER-DNA contains ER-responsive sequences (ER, PR, ps2 and c-myc). PCR analysis demonstrated that ER-DNA contains the ps2 gene promoter, which is expressed in ER positive cells. Recent evidence shows that ER and associated coactivators are recruited to the estrogen inducible promoters in a cyclical manner. It is reasonable to believe that ER interaction with DNA is dynamically altered during estrogen induction. ER-bound DNA from MCF-7 (T5) cells treated with estradiol (E2) for 30, 60 and 120 min were isolated and analyzed by PCR. A library of ER-bound DNA from 30 min E2 treated MCF-7 (T5) cells has been constructed. ChIP-ER-DNA fragments were cloned, sequenced, and blast searched in GenBank. Approximately 900 positive colonies were collected from this library, and approximately 100 cloned DNA fragments were sequenced. A $\frac{1}{2}$ (ERE)-Spl sequence has been identified in PACE4 (pro-protein convertase) and EWS (Ewing's sarcoma) genes. Studies to determine whether these genes respond to estrogen is currently being determined.			
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4. INTRODUCTION

A. ER responsive genes in human breast cancer cells

The proliferation of cells in hormonally dependent tumors is regulated by the expression of ER responsive genes. ER is a DNA-binding protein that can transcriptionally regulate target genes by directly interacting with the gene's promoter elements (Davie et al., 1999; Murphy, 1998). ER binds to an estrogen response element (ERE) consisting of a palindromic DNA sequence. ER interaction with the ERE results in structural changes in both the ER protein and ERE DNA (Wood et al., 1998). ER can also interact synergistically with several transcription factors and bind to a half ERE (Beato, 1989; Gorski et al., 1993; Meyer et al., 1989). Most of the characterized EREs of breast cancer estrogen responsive genes have ER and Sp1 binding sites (Xie et al., 1999; Qin et al., 1999). The regulatory regions of these genes (e.g. retinoic acid receptor α 1, cathepsin D, c-fos, adenine deaminase, hsp27, and insulin-like growth factor-binding protein-4 genes) have an $\frac{1}{2}$ (ERE) (N)x Sp1 (a half-site ERE positioned next to a Sp1 site) (Duan et al., 1998; Krishnan et al., 1994; Xie et al., 2000). The *c-myc* gene may be regulated by an ER/Sp1 interaction (Dubik and Shiu, 1992; Miller et al., 1996). The pS2 gene has a consensus ERE and an ERRE (ER related element, contains a half ERE site) in the promoter (Jeltsch et al., 1987; Lu et al., 2001). Three Sp1 binding sites in this region have been identified (2001 annual report, Fig.3). It is possible that Sp1 and ER have roles in regulating the transcriptional activity of the pS2 promoter.

Recently, a number of ER-responsive genes in breast cancer cells and breast carcinoma have been identified by SSH (suppression subtraction hybridization) and cDNA microarray analyses (Yang et al., 1999; Kuang et al., 1998; 't Veer et al., 2002; Sorlie et al., 2001). However, all of these reported ER-responsive genes are identified based on the increase in their mRNA levels, a result of direct or indirect transcriptional activation by ER. The ChIP protocol was designed to isolate ER-bound DNA *in situ*, where ER plays a role not only as an activator or a repressor, but perhaps also in chromatin structure.

B. RESEARCH OBJECTIVES

The goal of this research is to isolate and identify the ER-bound DNA in human breast cancer cells. In the first year, we had completed the majority of the proposed three tasks. For these studies we used a monoclonal antibody against human ER α that was specific and efficient in the immunoprecipitation and ChIP assays. The conditions for the ChIP assay were optimized, and the ER-DNA from ChIP was tested by Southern blotting and PCR analyses. In the second year ER-DNA from MCF-7 (T5) cells was isolated and inserted into a vector, pGEM-Easy, to construct a genomic DNA library. Approximately 900 positive clones were collected, and approximately 100 clones were sequenced and blast searched in GenBank. DNA sequences to which ER is bound *in situ* has been identified from the library. A $\frac{1}{2}$ (ERE)-Sp1 sequence has been identified in a region downstream (3') of exon 17 of the PACE4 (pro-protein convertase) gene and in a region upstream (5') of exon 16 of the EWS (Ewing's sarcoma) gene. Future studies will determine whether these genes respond to estrogens.

Task 1 Month 1-15 Large batch tissue culture of MCF-7 cells and MCF-7 cells that stably express epitope-tagged ER under the control of the tetracycline-on system

In the 2001 annual report, we provided evidence supporting the use of a monoclonal anti-ER α antibody to isolate DNA sequences bound to ER *in situ*. The efficiency and specificity of this antibody negated the need to use an epitope-tagged GFP-ER stably transferred MCF-7 cells (see 2001 annual report, page 11). At the time that time we had submitted this proposal we were uncertain as to the availability of an anti-ER α antibody suitable for our studies. Further, recent studies have revealed that expression of the epitope-tagged GFP-ER alters expression of endogenous genes in breast cancer cells (Zhao et al., 2002). As we do not want to perturb the cells, we decided to use the commercial antibody to isolate native ER.

Task 2 Month 2-15 Establish protocol to isolate DNA bound *in situ* to ER and epitope-tagged ER in above mentioned cell lines. Cells will be incubated with cisplatin or formaldehyde, DNA fragmented and ER-DNA isolated by immunoprecipitation. Determine efficiency of ER cross-linking to nuclear DNA in immunoblotting experiments.

In the first year, we have completed this task, established the ChIPs protocol and characterized the cross-linking procedure.

Task 3 Month 9-18 Characterization of ER-DNA by Southern and Northern blot analyses. Develop high resolution mapping protocol to find location of ER along the *c-myc* promoter.

We have analyzed ER-DNA isolated by ChIP by Southern blotting and PCR analysis (2001 annual report, page 14). pS2 is an estrogen inducible gene that is expressed in ER positive cells (Berry et al., 1989). In the 5'-flanking region of pS2 gene, there is an ERE (estrogen responsive element) and an ERRE (ER related element, contains a half ER binding site) (Jeltsch et al., 1987; Tora et al., 1989). Inspection of the pS2 promoter revealed several potential Sp1 sites positioned on either side of the ERE. We have identified three Sp1 binding sites close to the ERE and ERRE in the pS2 promoter using an EMSA assay (2001 annual report, pg. 13). Recent evidence shows that a number of ER responsive promoters, such as cathepsin D and *c-fos*, recruit ER-Sp1 complexes to regulate their transcription. The pS2 gene is expressed only in ER positive cells, and its promoter may be regulated by ER and Sp1. The *c-myc* gene is an E2 regulated gene in ER positive cells and constitutively expressed in ER negative cells. The *c-myc* promoter may be regulated by ER and Sp1. In this project, we validated the procedure by showing that ER-DNA isolated by ChIP had the E2 regulated

pS2 promoter sequence. We are currently testing if the putative ERE of the E2 regulated *c-myc* promoter is present in the ER-DNA.

In our statement of work, two new tasks (4 and 5) should be completed in the second year.

Task 4 Month 12-18 Identification of estrogen-inducible genes whose regulatory elements are bound directly or indirectly with ER *in situ* using DNA ChIPs.

Task 5 Month 12-24 PCR amplification and cloning of isolated DNA (ER-DNA) into pBluescript to generate a library of DNA sequences that are bound to ER and epitope-tagged ER *in situ*.

In this report we show the progress that we have made in achieving the above tasks. In the second year, we constructed a genomic DNA library that contains ER-DNA. ER-DNA was inserted into the vector pGEM-Easy, and the positive colonies were sequenced. Approximately 900 clones were collected, and approximately 100 clones have been sequenced and blast searched in GenBank. After analysis of the sequences, we found that several sequences were repeated with high frequency. To avoid repeatedly sequencing the same DNA, we have designed a new subtraction selection strategy to avoid repetitive sequencing of previously characterized ER-DNA sequences.

5. BODY REPORT

A. EXPERIMENTAL METHODS

i. Cell culture and cross-linking

Human breast cancer MCF-7 (T5) cells (ER-positive and hormone-dependent) and MDA MB 231 cells (ER-negative and hormone-independent) were grown as described previously (Miller et al., 1996; Sun et al., 2001). MCF-7 (T5) cells were grown in estrogen deplete or estrogen replete conditions as described before (Sun et al., 2001). Cells cultured under E2 deplete conditions were incubated at 37°C for 0, 30, 60 and 120 minutes with E2 (10 nM). The harvested cells were cross-linked in 1% formaldehyde for 10 minutes at room temperature. After washing, the cells were used for the ChIP experiments.

ii. Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation was performed as described previously (2001 annual report). Briefly, the cross-linked cells were resuspended in Lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) and incubated on ice for 10 minutes. The cells were sonicated with 4 sets of 10 second pulses at 30% output. Under these conditions the DNA fragment lengths ranged from 200 to 1000 base pairs. After a brief centrifugation, the supernatant was diluted to 2 A₂₆₀/ml with Dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl). A 1/200 dilution of anti-ER α mouse monoclonal antibodies (NOVOCASTRA, UK) was added to a 2 A₂₆₀ of chromatin fraction and incubated overnight at 4°C. Approximately 50 μ l of protein A sepharose slurry (1:1) was added and incubated at 4°C for 2 hours. The beads were washed sequentially with Low Salt Wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), High Salt Wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl Wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1) and twice with TE buffer, pH 8.0. Protein-DNA cross-links were reversed in 0.2 M NaCl in TE buffer at 65°C for 4 hours. DNA fragments were extracted with phenol / chloroform and precipitated with ethanol.

iii. Construction of library

ER-DNA fragments were either directly or with a linker inserted into vector pGEM-Easy (Promega). ER-DNA was ligated with a linker (GCGGTGACCCGGGAGATCTGAATT) as described previously (Sun et al., 1996). The DNA fragments with linker were amplified by PCR before insertion into the pGEM-Easy vector. Briefly, one µg of DNA from ChIP was incubated with linker in the presence of T4 DNA ligase (4 units) at 4°C overnight and amplified by PCR using a primer (GAATTCAAGTC). After 30 cycles (94°C 30 s, 55°C 30 s and 72°C 45 s), Ten µl of PCR product was ligated to pGEM-Easy. Bacteria (*E.coli*, DH5 α) were transformed with the plasmids as previously described (Sun et al., 1992).

iv. PCR

ER-DNA was analyzed by PCR to detect the pS2 promoter (P-U, 5'-GACGGAATGGGCTTCATGAGC, and P-L, 5'-GATAAACATTGCCTAAGGAGG). After 30 cycles (95°C 30 s, 56°C 30 s, 72°C 45 s), 20 µl of the PCR reaction was loaded onto a 1% agarose gel and the PCR products were visualized by staining with ethidium bromide.

v. ER-bound DNA cloning and sequencing

Two ml LB medium supplemented with ampicillin were inoculated with bacteria colonies. DNA was extracted and digested with EcoR1 and Not1. The restriction enzyme digestion was separated using 1% agarose gel and the DNA fragments were visualized by stained with ethidium bromide. The plasmids containing insert DNA were sequenced using the ABI Prism 310 Genetic Analyzer.

vi. Blast search of GenBank

The DNA sequence of the insert was submitted to GenBank (NIH) database and identical or similar sequences were identified by the Blast search program.

B. RESULT AND DISCUSSION

The strategy to generate a library of genomic DNA sequences bound to ER in human breast cancer cells is shown in a flow chart (Fig.1). This protocol contains three different steps: isolation of ER-DNA by ChIPs, construction of ER-DNA library and identify the ER-DNA sequences.

I. Analysis of ER-bound DNA from ChIP

Recently, Brown and colleagues reported evidence that ER and associated coactivators were recruited to the E2 inducible cathepsin D promoter in a cyclical manner (Shang et al., 2000). The loading of ER onto this promoter is highest at 30-45 min after E2 addition, with a second peak at 120-135 min. These data suggest that ER is directly or indirectly binding to DNA in a dynamic manner. To recover most, if not all, of ER-bound DNA induced by estradiol we carried out ChIP using MCF-7 (T5) cells incubated with estradiol for different periods.

The ER-DNA from MCF-7 (T5) cells incubated with 10 nM E2 for 0, 30, 60 and 120 minutes was isolated by ChIP. The ER-DNA was analyzed by PCR using primers to detect a pS2 promoter fragment. Figure 2 shows that in the absence of E2 low levels of ER reside on the pS2 promoter. After E2 induction at 30, 60 and 120 minutes, more ER was bound to the pS2 promoter. In contrast to E2, there was less binding of ER to the pS2 promoter when ICI 182,780.

Current studies suggest that the optimal time for the binding of ER to promoters of E2 responsive genes varies (Castro-Rivera et al., 2001; Shang et al., 2000). Thus, we will construct genomic DNA libraries that contain the ER-DNA from 30, 60 and 120 minutes E2 treated cells and identify the ER-DNA sequences.

2. Construction of ER-bound DNA library

To increase the amount of recovered ER-DNA, the ER-DNA fragments from ChIPs were amplified. We used a linker-ligation and PCR procedure to amplify ER-DNA and then inserted the amplified DNA into a vector, pGEM-Easy, to generate the library.

ER-DNA was ligated with a unique linker (see Material and Methods) and amplified by PCR. The PCR products were ligated into pGEM-Easy. *E.coli* (DH5 α) was transformed with the plasmids and the positive colonies were selected. DNA extracted from the positive colonies was digested with restriction enzymes, EcoR1 or Not 1, to analyze the inserted DNA. Fig. 3 shows that the ER-DNA with different sizes was inserted into the vector. The DNA cloned into the plasmid was sequenced. Initially we constructed a library containing ER-DNA from 30 minutes E2 treated MCF-7 (T5) cells. Next, we will construct several libraries with ER-DNA isolated by ChIPs from cells incubated with E2 for 60 and 120 minutes.

Approximately 900 positive clones were selected from the first library. The DNA fragments from the plasmids were between 1000 bp to 200 bp, in agreement with the DNA fragment size distribution after sonication.

3. Characterization of ER- DNA sequences

ER-DNA from the positive plasmids was sequenced using the fluorescent PCR procedure. Approximately 100 to 400 nucleotides of sequence could be read, depending on the quality of DNA and reaction conditions. Approximately 100 clones have been sequenced, however only 20 sequences could be read for stretches of 300 to 400 nuncleotides. Problems with the other sequences may be the presence of GC-rich sequences or secondary structure. We are currently optimizing the sequencing conditions to solve these problems.

The DNA sequences were used in a blast search using the SeqTaq program, produced by our Institute, to identify and remove plasmid sequences (the sequences from vector pGEM-Easy). The sequences containing only ER-DNA inserts were used in blast search of GenBank (NIH). The identified DNA sequences associated with ER *in situ* are listed in Table 1. The identified sequences did not contain the classical ERE, but contained a $\frac{1}{2}$ (ERE)-Sp1 sequence. Many of the sequenced clones had the EWS DNA sequence. The high representation of this sequence may be due to several reasons, including preferential growth in bacteria. To avoid re-sequencing this and other

characterized inserts, we will pre-screen the library to identify these sequences and characterize the remainder.

4. PACE4 may be an ER-responsive gene

The insert DNA sequence of ER-DNA clones was identified. One clone contained a sequence downstream of exon 17 of the PACE4 gene. Fig. 4 shows the sequence of PACE4 exon 17 (GenBank AB001909). Exon 17 sequence is from nucleotides 750 to 1789, and it contains a poly A signal (Fig. 6). The ER-DNA sequence was found to match a 246 nucleotide region approximately 1 kb downstream from exon 17 of PACE4. A $\frac{1}{2}$ (ERE)-Sp1 was found in this region, suggesting that ER associates with this region *in situ*. It remains to be determined that PACE4 is an E2 responsive gene.

(Rousseau et al., 1993)PACE4 is a mammalian pro-protein convertase family member and plays an important role in tumorigenesis (Cheng et al., 1997; Bassi et al., 2000). An analyses of breast tumors revealed that PACE4 is primarily expressed in ER positive but not ER negative breast epithelial cancer cells (Cheng et al., 1997). This observation is congruent with the possibility that PACE4 is an E2 responsive gene. We will further characterize the regulation of PACE4 by ER. Northern blotting and RT-PCR analyses will determine whether PACE4 is an E2 responsive gene.

5. ER is bound to the EWS gene

Several ER-DNA clones contained the sequence to the intron 16 of the EWS gene. EWS (Ewing's sarcoma) gene consists of 17 exons. Fig. 5 shows the sequence of intron 16 and exon 17. A 238 ER-DNA nucleotide sequence matched a sequence in intron 16, which harbored a $\frac{1}{2}$ (ERE)-Sp1 sequence (Fig. 6). The EWS gene is involved in oncogenesis in a number of cancer cells and tumors (Kovar et al., 2001; Rossow and Janknecht, 2001). The presence of a $\frac{1}{2}$ (ERE)-Sp1 sequence suggests that EWS is an E2 regulated gene. We are currently doing studies to determine if this is true.

6. Pre-screening of the ER-DNA library

To avoid re-sequencing previously characterized DNA sequences, the library will be pre-screened to set aside previously characterized sequences. The DNA fragments characterized from the last batches of clones, such as inserts containing PACE4 and EWS sequenced, will be used as probes to detect these sequences in the other clones which will be dotted on a Nylon membrane using a slot blot manifold. The membrane will be hybridized with labeled DNA fragments to previously characterized inserts. After hybridization, the positive clones will be set aside and the remainder of the clones processed.

D. RECOMMENDATIONS

Progress in the past year is on target with our proposed SOW. ER-DNA from E2 treated MCF-7 (T5) cells has been isolated by ChIP procedure. To recover most, if not all, ER-DNA isolated from MCF-7 (T5) cells the E2 treatment has been carried out for the different times. These DNA have been tested by PCR analysis, and it has been shown that these DNA fragments contain the E2 responsive pS2 promoter fragment. We have constructed an ER-DNA library from the 30 minutes E2 treated MCF-7 (T5) cells and approximately 900 positive clones have been collected. Approximately 100 clones have been sequenced. Several genes contained a $\frac{1}{2}$ (ERE)-Sp1 binding sequence. Future experiments will use a pre-screening strategy to avoid re-sequencing previously characterized clones, allowing us to focus on the sequence of other ER-bound DNA. Several libraries containing ER-DNA from MCF-7 (T5) cells induced by E2 for 60 and 120 minutes will be constructed.

6. KEY RESEARCH ACCOMPLISHMENTS

Isolation and identification of ER-bound DNA sequences from human breast cancer MCF-7 (T5) (ER positive and hormone dependent) cells.

7. REPORTABLE OUTCOMES

A. Abstracts:

Jian-Min Sun, Hou Yu Chen and James R. Davie, Isolation and characterization of estrogen responsive genes in human breast cancer cells, Era of Hope 2002 meeting.

B. Publications:

1. Jian-Min Sun, Hou Yu Chen, Mariko Moniwa, David W. Litchfield, Edward Seto, and James R. Davie, The transcriptional repressor Sp3 is associated with CK2 phosphorylated histone deacetylase 2. *J.Biol.Chem.* 277:35783-35786, 2002
2. James R. Davie, Inhibition of histone deacetylase activity by butyrate. *J. Nutrition*, in press.
3. Virginia A. Spencer, Jian-Min Sun, Lin Li, and James R. Davie, Chromatin Immunoprecipitation as a tool for the study of histone acetylation and transcription factor binding, submitted to Methods
4. Jian-Min Sun, Virginia Spencer, Hou Yu Chen, Lin Li and James R. Davie, Sub-cellular fractionation and assays for studying histone deacetylases and histone acetyltransferases, submitted to Methods

8. CONCLUSION

We have applied a chromatin immunoprecipitation assay with anti-ER monoclonal antibodies to isolate ER-bound DNA fragments from MCF-7 (T5) breast cancer cells treated with estradiol for 30, 60 and 120 minutes. This library should include the majority of DNA sequences bound directly or indirectly to ER *in situ*. We have constructed an ER-DNA library from 30 minute E2 treated MCF-7 (T5) cells. Approximately 900 clones were isolated and of these 100 clones have been characterized. Submission of several sequences to GenBank using the Blast Search program identified the PACE4 and EWS genes. Both genes have a putative $\frac{1}{2}$ (ERE)-Sp1 regulatory element.

9. REFERENCES

- 't Veer,L.J., Dai,H., van de Vijver,M.J., He,Y.D., Hart,A.A., Mao,M., Peterse,H.L., van der,K.K., Marton,M.J., Witteveen,A.T., Schreiber,G.J., Kerkhoven,R.M., Roberts,C., Linsley,P.S., Bernards,R., and Friend,S.H. (2002). Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 415, 530-536.
- Bassi,D.E., Mahloogi,H., and Klein-Szanto,A.J. (2000). The proprotein convertases furin and PACE4 play a significant role in tumor progression. *Mol. Carcinog.* 28, 63-69.
- Beato,M. (1989). Gene regulation by steroid hormones. *Cell* 56, 335-344.
- Berry,M., Nunez,A.M., and Chambon,P. (1989). Estrogen-responsive element of the human pS2 gene is an imperfectly palindromic sequence. *Proc. Natl. Acad. Sci. U. S. A.* 86, 1218-1222.
- Castro-Rivera,E., Samudio,I., and Safe,S. (2001). Estrogen regulation of cyclin D1 gene expression in ZR-75 breast cancer cells involves multiple enhancer elements. *J. Biol. Chem.* 276, 30853-30861.
- Cheng,M., Watson,P.H., Paterson,J.A., Seidah,N., Chretien,M., and Shiu,R.P. (1997). Pro-protein convertase gene expression in human breast cancer. *Int. J. Cancer* 71, 966-971.
- Davie,J.R., Samuel,S.K., Spencer,V.A., Holth,L.T., Chadee,D.N., Peltier,C.P., Sun,J.-M., Chen,H.Y., and Wright,J.A. (1999). Organization of chromatin in cancer cells:role of signalling pathways. *Biochem. Cell Biol.* 77, 265-275.
- Duan,R., Porter,W., and Safe,S. (1998). Estrogen-induced c-fos protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor Sp1 complex formation. *Endocrinology* 139, 1981-1990.
- Dubik,D. and Shiu,R.P. (1992). Mechanism of estrogen activation of c-myc oncogene expression. *Oncogene* 7, 1587-1594.
- Gorski,J., Furlow,J.D., Murdoch,F.E., Fritsch,M., Kaneko,K., Ying,C., and Malayer,J.R. (1993). Perturbations in the model of estrogen receptor regulation of gene expression. *Biol. Reprod.* 48, 8-14.
- Jeltsch,J.M., Roberts,M., Schatz,C., Garnier,J.M., Brown,A.M., and Chambon,P. (1987). Structure of the human oestrogen-responsive gene pS2. *Nucleic Acids Res.* 15, 1401-1414.
- Kovar,H., Jug,G., Hattinger,C., Spahn,L., Aryee,D.N., Ambros,P.F., Zoubek,A., and Gadner,H. (2001). The EWS protein is dispensable for Ewing tumor growth. *Cancer Res.* 61, 5992-5997.

Krishnan,V., Wang,X., and Safe,S. (1994). Estrogen receptor-Sp1 complexes mediate estrogen-induced cathepsin D gene expression in MCF-7 human breast cancer cells. *J. Biol. Chem.* 269, 15912-15917.

Kuang,W.W., Thompson,D.A., Hoch,R.V., and Weigel,R.J. (1998). Differential screening and suppression subtractive hybridization identified genes differentially expressed in an estrogen receptor- positive breast carcinoma cell line. *Nucleic Acids Res.* 26, 1116-1123.

Lu,D., Kiriyama,Y., Lee,K.Y., and Giguere,V. (2001). Transcriptional regulation of the estrogen-inducible pS2 breast cancer marker gene by the ERR family of orphan nuclear receptors. *Cancer Res.* 61, 6755-6761.

Meyer,M.E., Gronemeyer,H., Turcotte,B., Bocquel,M.T., Tasset,D., and Chambon,P. (1989). Steroid hormone receptors compete for factors that mediate their enhancer function. *Cell* 57, 433-442.

Miller,T.L., Jin,Y., Sun,J.-M., Coutts,A.S., Murphy,L.C., and Davie,J.R. (1996). Analysis of human breast cancer nuclear proteins binding to the promoter elements of the c-myc gene. *J. Cell. Biochem.* 60, 560-571.

Murphy,L.C. (1998). Mechanisms of hormone independence in human breast cancer. *In Vivo.* 12, 95-106.

Qin,C., Singh,P., and Safe,S. (1999). Transcriptional activation of insulin-like growth factor-binding protein-4 by 17beta-estradiol in MCF-7 cells: role of estrogen receptor- Sp1 complexes. *Endocrinology* 140, 2501-2508.

Rosso,K.L. and Janknecht,R. (2001). The Ewing's sarcoma gene product functions as a transcriptional activator. *Cancer Res.* 61, 2690-2695.

Rousseau,S., Asselin,M., Renaud,J., and Ruiz-Carrillo,A. (1993). Transcription of the histone H5 gene is regulated by three differentiation-specific enhancers. *Mol. Cell. Biol.* 13, 4904-4917.

Shang,Y., Hu,X., DiRenzo,J., Lazar,M.A., and Brown,M. (2000). Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell* 103, 843-852.

Sorlie,T., Perou,C.M., Tibshirani,R., Aas,T., Geisler,S., Johnsen,H., Hastie,T., Eisen,M.B., van de,R.M., Jeffrey,S.S., Thorsen,T., Quist,H., Mateise,J.C., Brown,P.O., Botstein,D., Eystein,L.P., and Borresen-Dale,A.L. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc. Natl. Acad. Sci. U. S. A* 98, 10869-10874.

Sun,J.-M., Chen,H.Y., and Davie,J.R. (2001). Effect of estradiol on histone acetylation dynamics in human breast cancer cells. *J. Biol. Chem.* 276, 49435-49442.

Sun,J.-M., Ferraiuolo,R., and Davie,J.R. (1996). In situ footprinting of chicken histone H5 gene in mature and immature erythrocytes reveals common factor binding sites. *Chromosoma* 104, 504-510.

Sun,J.-M., Penner,C.G., and Davie,J.R. (1992). Analysis of erythroid nuclear proteins binding to the promoter and enhancer elements of the chicken histone H5 gene. *Nucleic Acids Res.* 20, 6385-6392.

Tora,L., Mullick,A., Metzger,D., Ponglikitmongkol,M., Park,I., and Chambon,P. (1989). The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties. *EMBO J.* 8, 1981-1986.

Wood,J.R., Greene,G.L., and Nardulli,A.M. (1998). Estrogen response elements function as allosteric modulators of estrogen receptor conformation. *Mol. Cell Biol.* 18, 1927-1934.

Xie,W., Duan,R., Chen,I., Samudio,I., and Safe,S. (2000). Transcriptional activation of thymidylate synthase by 17beta-estradiol in MCF-7 human breast cancer cells. *Endocrinology* 141, 2439-2449.

Xie,W., Duan,R., and Safe,S. (1999). Estrogen induces adenosine deaminase gene expression in MCF-7 human breast cancer cells: role of estrogen receptor-Sp1 interactions. *Endocrinology* 140, 219-227.

Yang,G.P., Ross,D.T., Kuang,W.W., Brown,P.O., and Weigel,R.J. (1999). Combining SSH and cDNA microarrays for rapid identification of differentially expressed genes. *Nucleic Acids Res.* 27, 1517-1523.

Zhao,H., Hart,L.L., Keller,U., Holth,L.T., and Davie,J.R. (2002). Characterization of stably transfected fusion protein GFP-estrogen receptor- in MCF-7 human breast cancer cells. *J. Cell Biochem.* 86, 365-375.

10. APPENDICES

Table 1. LIST of ER-BOUNDED DNA from ChIP

1, PACE4		Exon 16 – 3'
	$\frac{1}{2}$ (ERE)-Sp1	<u>GGTAAGCGGCAGGG</u>
2, EWS		5' - Exon 17
	$\frac{1}{2}$ (ERE)-Sp1	<u>ACTGGAAAGCGGGC</u>
3, HNC (Human normal cartilage)		cDNA-3'
	$\frac{1}{2}$ (ERE)-Sp1	<u>ACTGGGAAAACCCTGGCGTTA</u>
4, CPE (Carboxypeptidase E)		
	$\frac{1}{2}$ (ERE)	<u>GGTCA</u>
5, BcL2		Exon 2- 3'
	$\frac{1}{2}$ (ERE)-Sp1	<u>CCCTGCCGCTTACCC</u>

Table 1. List of characterized DNA sequences from the ER-DNA library. Several genes contained the $\frac{1}{2}$ (ERE)-Sp1 regulatory element.

Figure agenda:

Fig.1. The strategy of isolation and identification of ER-associated DNA sequences in human breast cancer cells. The procedure includes three major steps. The first step is to isolate ER-DNA from human breast cancer cells using the ChIP assay. The second step is to construct an ER-DNA library by inserting ER-DNA into the vector, pGEM-Easy. The third step is to characterize ER-DNA by sequencing and Blast searching against sequences in GenBank.

Fig.2. PCR analysis of ER-DNA. ER cross-linked to genomic DNA isolated from MCF-7 (T5) cells treated with 10 nM E2 for 0, 30, 60 and 120 min or ICI for 120 min, and MDA MB 231 cells was immunoprecipitated by anti-ER antibodies. Two primers (P-U and P-L) were designed to detect the pS2 promoter which has the ERE and ERRE sequences (Panel A). Two hundred ng of ER-DNA was used in each PCR reaction as template and 1 pM of pS2 primers were added in 50 μ l reaction. Twenty μ l of PCR product was electrophoretically resolved on a 1% agarose gel which was subsequently stained with ethidium bromide. PCR analysis of ER-DNA from MCF-7 (T5) cells incubated with E2 for 30 (panel B), 60 (panel C) and 120 minutes (pPanel D) is shown.

Fig.3. ER-DNA from 30 minute E2 treated cells was ligated into the vector pGEM-Easy. The bacteria (*E.Coli*, DH5 α) were transformed with the plasmids, and the isolated plasmids were digested with EcoR1 and Not1. DNA was separated on a 1% agarose gel and the gel was subsequently stained with ethidium bromide.

Fig.4. Sequence of PACE4 (pro-protein convertase) exon-16. Exon 16 occupies a sequence from 751 to 1788, and the polyadenylation sequence is at the end of exon16. A $\frac{1}{2}$ (ERE)-Sp1 sequence is found 1 kb downstream from exon 16. PACE4 is a potential E2 responsive gene.

Fig.5. Sequence of EWS (Ewing's sarcoma) exon 17. A $\frac{1}{2}$ (ERE)-Sp1 sequence occurs upstream of exon 17.

Fig.6. Diagram of ER-DNA containing PACE4 and EWS DNA sequences. A $\frac{1}{2}$ (ERE)-Sp1 regulatory element is in the ER-DNA sequence.

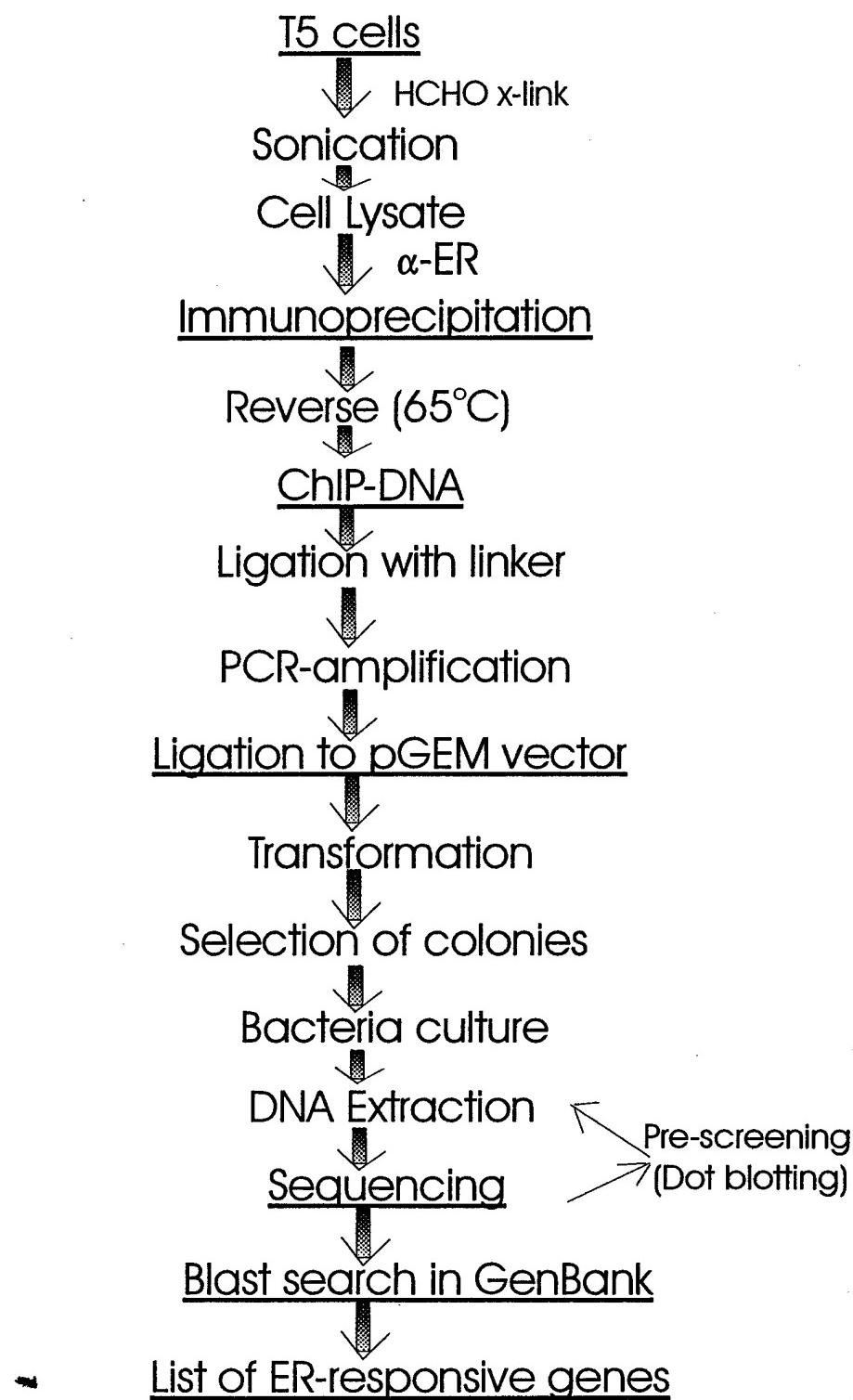


Fig.1.

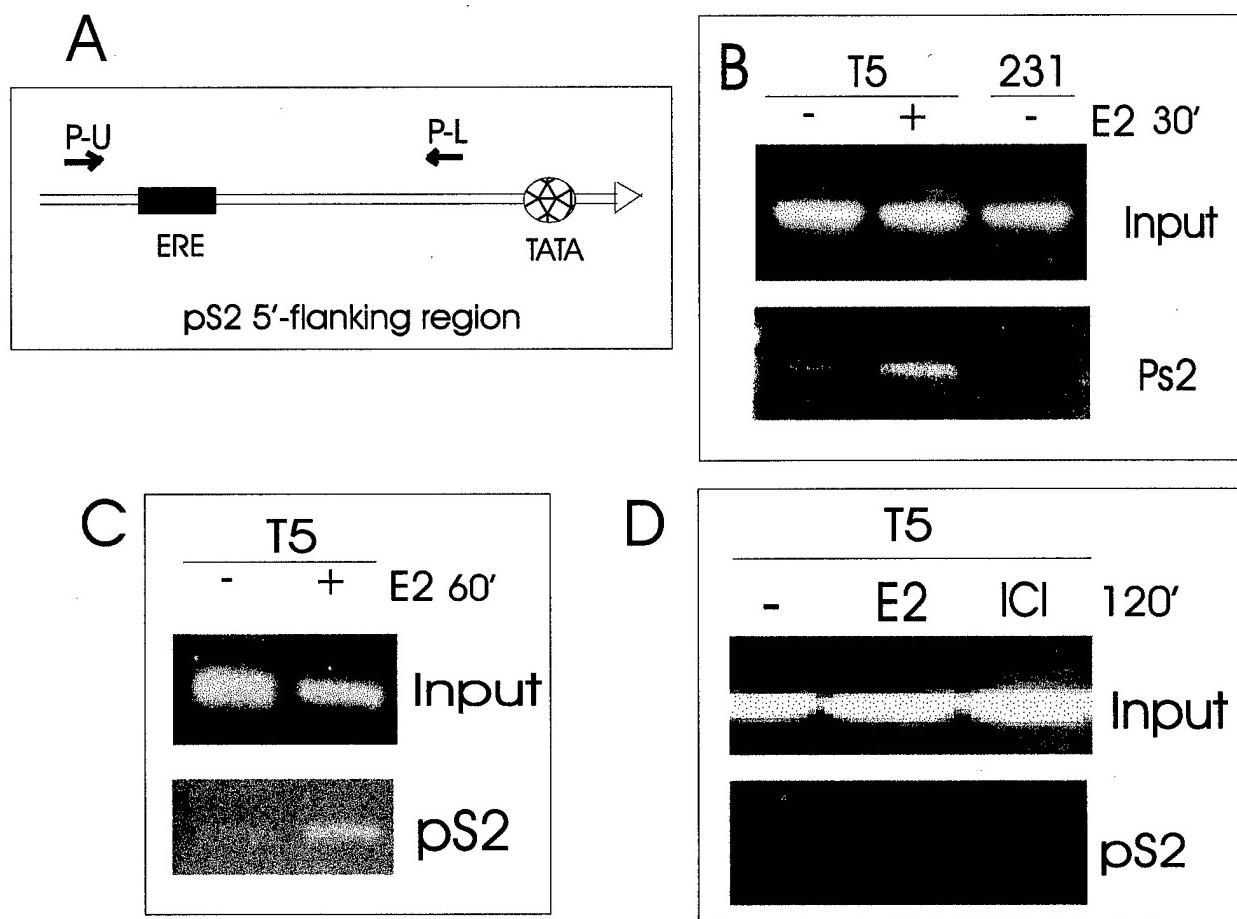


Fig.2.

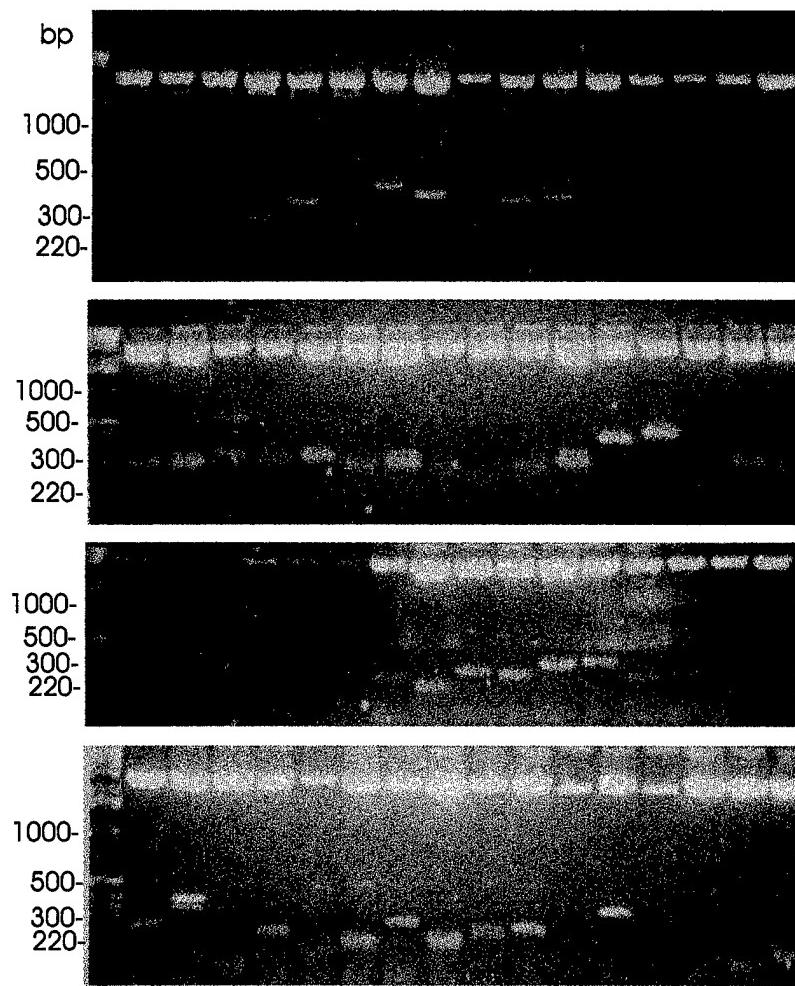


Fig.3.

PACE4 (pro-protein convertase), exon 16

(GenBank AB001909, GI:2281764)

751

[REDACTED]

1801 tgacagatct gaggaaaggagg gagagctctc ttgaaaacccc tttcccatat ttgcctcag
1861 ttttaggaat gaggtacgct tggcattcc tggtcctgc atggttcgcc attctggcaa
1921 gccagggtcg ccccagaata gataagtgtt tttgattttc gaacatttga cttaattact
1981 tatttccaga aacttcgtgt tcaactacgt ctctacaggc atttctactt ggtcttttc
2041 tccccctgaca tttcacagag tcctaactgt gctgcattgtta taacttgttc ttcttttg
2101 ttactgcata gcaagcatgt ttctatgtca taaaatatcct ttgtaaacag ctttttatag
2161 atttttata atactncacc aacagaagca ccatattta tttaagcatc tctctattgt
2221 tggacatgtt gattgctttc catttttcc tattatgagc ggacagcttta gtacaaaaag
2281 cctnctctg aattttaaat tatttcttttggattattca ccagaagtaa aattactggg
2341 ataaataaga ggtggaaacc acaaataatgc ctgccttga tacatattgc caatttgctt
2401 ttccaaatag ttgaactgtt ttgttagtctt accaggagtg ggcagttctg gaggaggtag
2461 atgataccct tccttaactt acattgtgtt cattactaac aaaggtgaac cttttncaa
2521 atgtttgtgc actgtgtttt ttcttttggaa aattgccctt tctggcgtgg tgctcacnc
2581 ttgtatccca aacnctttgg gaggccgagg caggnngato acctgaggc aggagtttga
2641 gaccancctg gccaaacagg gtgaaacccc gcctctacca aaaataaaaa aattagccgg
2701 tcatgggtt ggggcctat aatcccagct actcaggagg ctgaggcagg agaatcactt
2761 gaacccggga ggccggagggtt gcagtgagcc gagatttgc cacctcaactc cagcctggat
2821 gacagaaacn agtccgtct caaaaaaaaaaag aaaaaaaaaaattccctttt gnctttcneg
2881 agagcgcnc a gtgtgttat caagctgata agtgcgggtcg ggctqaacgg gggttgcgtq
2941 cacacaccccc anctggaggc gaacgaccta caccgaaactc agataacctac agcgtgagct
3001 atgagaaaaqc qccacqcttc ccgaaaggaaq aaaggccqac aggtatccgg **taagccggcag**
3061 ~~atcgaaaca~~ ggagagcqca cqagggagct tccaggggaa aacccctggat atctttataag
3121 tccctgtcggtt ttcggccacc tctgacttga qcotcggqca tgc

Exon 16:

ER-bound DNA: 2915-3160

½(ERE)-Sp1 site: 3049-3062

Fig.4.

EWS (Ewing's sarcoma) gene, exon 17

(GenBank: X73004, GI: 485853)

1 [REDACTED] attttcg ctttcttggg gaaaaccaac gtggaccgct tgcttggaaac tctctnaggg
61 ccagncntg aaggcaatc agctgttgc cgtctcaactg gtgaaaagaa aaaccaccnt
121 ggcgcccaaat acgaaaaccg cctctccccg cgcgttggcc gattcattaa tgcatcgcc
181 acgacaggtt tcccg**actgg** aaag**ggggca** **gt**gagcgcaa cgcaattaat gtgagttac
241 tcactcatta ggcccccag gctttacact ttatgcttcc ggctcgatg ttgtgtggaa
301 ttgtgagcgg ataacaattt cacacaggaa acagctatga ccatgattac gccaagcttg
361 tgatagtggt tgggaggagc caggaagggg caccctgggg ctctggaaagg gcttcctcac
421 cccttcccat tctaacccaa gggccctt tacctt [REDACTED]
481 [REDACTED] atgcagagac cccgcagago tgcattgact accagattt

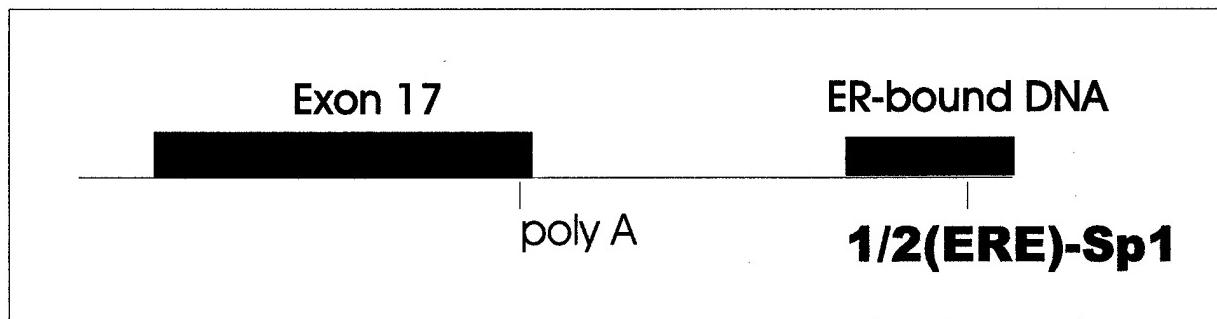
Exon 17: [REDACTED]

ER-bound DNA: 121-358

½(ERE)-Sp1: 195-213

Fig.5.

PACE4 gene



EWS gene

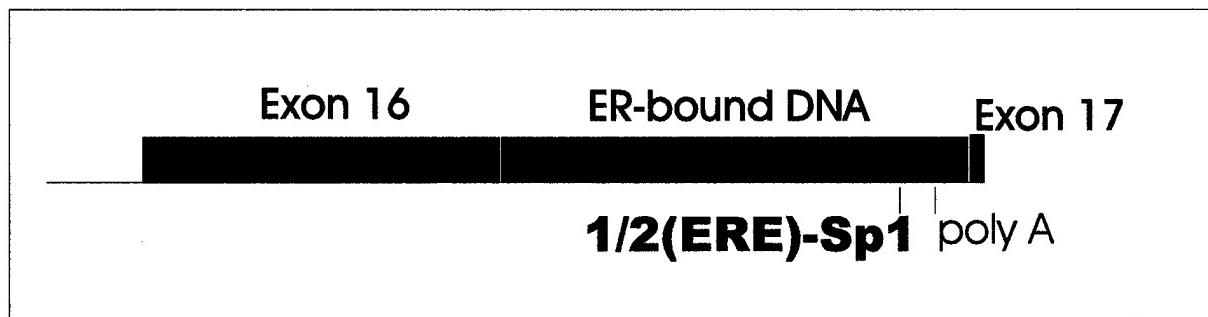


Fig.6.

The Transcriptional Repressor Sp3 Is Associated with CK2-phosphorylated Histone Deacetylase 2*

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Sp1 and Sp3 are ubiquitously expressed mammalian transcription factors that function as activators or repressors. Although both transcription factors share a common domain involved in forming multimers, we demonstrate that Sp1 and Sp3 form separate complexes in estrogen-dependent human breast cancer cells. Sp1 and Sp3 complexes associate with histone deacetylases (HDACs) 1 and 2. Although most HDAC2 is not phosphorylated in the breast cancer cells, HDAC2 bound to Sp1 and Sp3 and cross-linked to chromatin *in situ* is highly enriched in a phosphorylated form that has a reduced mobility in SDS-polyacrylamide gels. We show that protein kinase CK2 is associated with and phosphorylates HDAC2. Alkaline phosphatase treatment of HDAC2 and Sp1 and Sp3 complexes reduced the associated HDAC activity. Protein kinase CK2 is up-regulated in several cancers including breast cancer, and Sp1 and Sp3 have key roles in estrogen-induced proliferation and gene expression in estrogen-dependent breast cancer cells. CK2 phosphorylation of HDAC2 recruited by Sp1 or Sp3 could regulate HDAC activity and alter the balance of histone deacetylase and histone acetyltransferase activities and dynamic chromatin remodeling of estrogen-regulated genes.

Remodeling of chromatin structure mediated by ATP-driven chromatin-remodeling complexes and histone-modifying enzymes has a crucial role in gene expression. Acetylation of the core histones favors decondensation of the chromatin fiber by preventing interfiber interactions, whereas the unacetylated

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histone state contributes to chromatin condensation (1, 2). Dynamic histone acetylation catalyzed by histone deacetylases (HDAC)¹ and histone acetyltransferases allows the chromatin fiber to rapidly oscillate from the condensed and decondensed states (3, 4). In mammalian cells three classes of HDACs are identified. Class I HDACs, such as HDAC1 and HDAC2, are homologous to yeast RPD3, whereas class II HDACs are similar to yeast HDA1. Class III HDACs are related to yeast SIR2 (5, 6). HDAC1 and -2 are components of large multisubunit complexes called Sin3 or NuRD, which are recruited by transcriptional factors such as Mad, YY1, and Rb (5, 7–9).

Mammalian cells ubiquitously express Sp1 and Sp3. Sp3 has three isoforms, a long (L-Sp3) and two short forms (M1-Sp3, M2-Sp3) that are the products of differential translational initiation (10). Sp3 may act as a repressor or an activator, with the short forms acting only as repressors (10). The protein structure of L-Sp3 is very similar to that of Sp1, except that Sp3 has a repression domain located N-terminal to the zinc finger DNA-binding domain (11). It has been reported that the relative levels of Sp3 forms change with differentiation, with the differentiated Caco-2 cells expressing more long than short forms (12). Further, alterations in the relative levels of Sp1 to Sp3 have been recorded, with Sp3 levels being greater than Sp1 in primary keratinocytes (13).

In this study we investigated the association of histone deacetylase with Sp3 in human breast cancer cells. We found that Sp3 and Sp1 were associated with HDAC1 and a modified form of HDAC2. HDAC2 is shown to be associated with protein kinase CK2 and phosphorylated by this enzyme. The low abundance phosphorylated form of HDAC2 is preferentially associated with chromatin.

EXPERIMENTAL PROCEDURES

Cells and Plasmid—Human breast cancer T5 cells, estrogen receptor-positive and hormone-dependent, were grown in Dulbecco's modified Eagle's medium and 5% fetal bovine serum as previously described (4). T5 cells were grown under estrogen-depleted conditions, and in some cases estradiol was added for 20 min as reported (4). Plasmid pGST-HDAC2 has been described previously (14).

Cisplatin and Formaldehyde Cross-linking—T5 cells were incubated with 1 mM cisplatin at 37 °C for 2 h or with 1% formaldehyde at room temperature for 10 min as described previously (3, 15). The methods for isolating the proteins cross-linked to DNA *in situ* are described in detail (16, 17). Briefly, following cross-linking, cells were washed twice with TNM buffer (100 mM NaCl, 300 mM sucrose, 10 mM Tris-HCl, pH 8.0, 2 mM MgCl₂, 1% thioglycol) containing 1 mM PMSF, phosphatase inhibitors (25 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate) and protease inhibitor mixture (Roche Molecular Biochemicals). The cells were resuspended in cross-linking lysis buffer (5 M urea, 2 M guanidine hydrochloride, 2 M NaCl, and 0.2 M potassium phosphate buffer, pH 7.5) containing 1 mM PMSF, and the lysate was incubated with prehydrated hydroxyapatite (Bio-Rad). DNA-protein cross-links were reversed, and proteins were isolated.

Immunoprecipitation—T5 cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA) containing 1 mM PMSF, phosphatase inhibitors, and protease inhibitors mixture. The cells were sonicated twice for 15 s. The cell lysate was collected by centrifugation at 10,000 × g for 10 min at 4 °C and incubated with anti-Sp1 or anti-Sp3 antibodies for 16 h at 4 °C. The

¹ The abbreviations used are: HDAC, histone deacetylase; CK2, protein kinase CK2; L-Sp3, long form of Sp3; M1- and M2-Sp3, short forms of Sp3; PMSF, phenylmethylsulfonyl fluoride; GST, glutathione S-transferase; IP, immunoprecipitation; ID, immunodepletion.

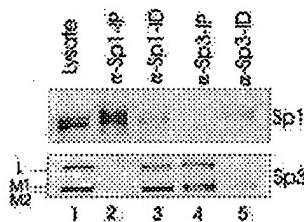


Fig. 1. Sp1 is not associated with Sp3. Two A_{260} of T5 cell lysate were incubated with 4 μg of anti-Sp1 antibodies, and the immunoprecipitation (IP, lane 2) and immunodepletion (ID, lane 3) fractions were collected. The immunodepleted fraction was next incubated with anti-Sp3 antibodies, yielding IP (lane 4) and ID (lane 5) fractions. Ten μl of cell lysate (lane 1), IP, and ID fractions were loaded onto a SDS-10% polyacrylamide gel, transferred to nitrocellulose membranes, and immunochemically stained with anti-Sp1 and anti-Sp3 antibodies. The long (L) and short (M1 and M2) forms of Sp3 are identified.

beads were washed four times with 5 volumes of immunoprecipitation buffer and frozen at -80°C .

Sequential Immunoprecipitations—Sequential immunoprecipitations were done as described above. Briefly, cell lysates were incubated with anti-Sp1 antibodies. The immunoprecipitated and immunodepleted (supernatant) fractions were collected. Secondary immunoprecipitations were done with anti-Sp3 antibodies, and the immunoprecipitated and immunodepleted fractions were collected.

Immunoblot Analysis—Immunoblot analysis was carried out as described previously (4). Polyclonal antibodies against human HDAC1 (Affinity Bioreagents Inc. (ABR)), HDAC2 (ABR), HDAC3 (ABR), Sp1 (Santa Cruz Biotechnology Inc.), and Sp3 (Santa Cruz) were used. Polyclonal antibodies against CK2 α and α' were described previously (18). Quantification of proteins on immunoblots was done as described previously (19).

HDAC Activity Assay—HDAC activity assays were performed as reported previously (20).

Protein Phosphatase Digestion—Immunoprecipitated fractions and DNA cross-linked protein fractions were incubated with or without calf intestinal alkaline phosphatase (Amersham Biosciences) at 37°C for 1 h. The protein was separated on SDS-12% polyacrylamide gels and transferred onto nitrocellulose membrane for immunochemical staining.

Protein Kinase CK2 Assay—Purified GST-HDAC2 was incubated in a buffer consisting of 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 25 mM β -glycerophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM PMSF, and 100 μM ATP solution containing [γ -³²P]ATP at a final concentration of 1 $\mu\text{Ci}/\mu\text{l}$. The reaction tube was preincubated at 30°C for 10 min before the addition of purified CK2 (from bovine testis as described previously (18, 21)). The reaction was incubated for 10 min before termination upon the addition of SDS sample buffer. The sample was boiled for 5 min and loaded into a SDS-15% polyacrylamide gel. After electrophoresis, the gel was dried and autoradiographed.

RESULTS AND DISCUSSION

Sp1 and Sp3 contain a similar D domain that is required for the proteins to form multimers (22–24). *In vitro* evidence suggests that Sp1 and Sp3 may form heteromultimers (24). Sequential immunoprecipitations and immunoblotting experiments tested whether Sp1 associated with Sp3 *in situ* in T5 human breast cancer cells. A T5 cell lysate was incubated with anti-Sp1 antibodies, and the immunoprecipitate was collected. The immunodepleted supernatant was next incubated with anti-Sp3 antibodies, and the immunoprecipitate was harvested. Fig. 1 shows the analyses of the fractions immunochemically stained with anti-Sp3 or anti-Sp1 antibodies. Anti-Sp1 antibodies efficiently immunoprecipitated Sp1 but not Sp3 (lane 2), whereas antibodies against Sp3 immunoprecipitated Sp3 but not Sp1 (lane 4). The results demonstrated that Sp1 does not associate with Sp3 in T5 cells. Further, the immunoblot results show that T5 breast cancer cells express the L-, M1-, and M2-Sp3 forms, with the latter lower molecular mass forms of Sp3 predominating. Quantification of Sp1 and Sp3 levels on immunoblots indicated that Sp1 was 3-fold more abundant than Sp3.

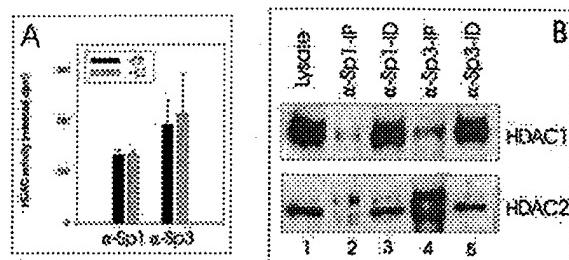


Fig. 2. Sp1 and Sp3 are associated with HDAC1 and HDAC2. Two A_{260} of T5 cell lysate treated with (+E2) or without (-E2) 10 nM estradiol was immunoprecipitated using 4 μg of anti-Sp1 and anti-Sp3 antibodies. HDAC activities of immunoprecipitated fractions were analyzed using ³H-labeled histones (A). Each value represents the mean \pm S.E. of three different preparations. In B, 10 μl of cell lysate, IP, and ID fractions prepared as described in Fig. 1 were loaded onto a SDS-10% polyacrylamide gel, transferred to nitrocellulose membranes, and immunochemically stained with anti-HDAC1 and HDAC2 antibodies.

It has been reported that Sp1 recruits HDAC1 to repress transcription (25). It is conceivable that Sp3 also recruits HDAC to repress transcription. Fig. 2A shows that Sp1 and Sp3 were associated with HDAC activity. Culturing breast cancer cells in the absence or presence of estradiol did not affect the HDAC activity associated with these transcription factors. The sequential immunoprecipitation strategy with anti-Sp1 followed by anti-Sp3 antibodies was applied to decide which of the HDACs was bound to Sp1 or Sp3. Fig. 2B shows that Sp1 and Sp3 were associated with HDAC1 and HDAC2. Both immunoprecipitates were highly enriched in a slower migrating form of HDAC2. Neither Sp1 nor Sp3 was associated with HDAC3 (data not shown).

The preferential association of the slower migrating form of HDAC2 with the transcription factors Sp1 and Sp3 suggested that this form of HDAC2 may be selectively in contact with chromatin. T5 cells were incubated with the cross-linker cisplatin, and the proteins cross-linked to nuclear DNA *in situ* were isolated. Unlike formaldehyde, cisplatin does not form protein-protein cross-links. Fig. 3A shows that the slower migrating form of HDAC2 was enriched in the proteins cross-linked to DNA. Similar results were obtained with the cross-linker formaldehyde. HDAC3 was not cross-linked to nuclear DNA with cisplatin.

As HDAC1 is phosphorylated (26, 27), we determined whether the slower migrating form of HDAC2 was phosphorylated by incubating the proteins cross-linked to DNA with alkaline phosphatase. Fig. 3B demonstrates that incubation of the protein sample with alkaline phosphatase resulted in the disappearance of the slower migrating form of HDAC2 and the appearance of a band co-migrating with the major HDAC2 band present in the cell lysate. Identical results were obtained when the Sp1 or Sp3 immunoprecipitates were incubated with alkaline phosphatase (data not shown). The phosphorylated form of HDAC1 has a reduced mobility on SDS gels (27). However, we did not observe an enrichment of the slower migrating HDAC1-phosphorylated form in the Sp1 or Sp3 immunoprecipitates or in the protein fraction cross-linked to DNA with cisplatin. Treatment of these fractions with alkaline phosphatase did not alter the mobility of HDAC1, ruling out the possibility that the entire HDAC1 population was phosphorylated.

Recently, HDAC1 was shown to be phosphorylated by CK2 (26, 27). The CK2 phosphorylation sites located in the C-terminal region of HDAC1 are conserved in HDAC2 (27). Fig. 4A illustrates that incubation of HDAC2 in the cell lysate with CK2 and ATP generates the slower migrating HDAC2 form. Inhibition of CK2 with apigenin prevented the appearance of this band. Further, GST-HDAC2 and casein but not GST were radiolabeled with CK2 and [³²P]ATP (Fig. 4B). To decide if CK2

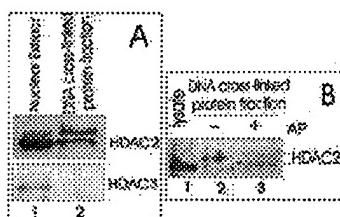


FIG. 3. Phosphorylated HDAC2 is preferentially associated with chromatin. T5 cells were incubated with 1 mM cisplatin, and proteins cross-linked to DNA were isolated. Ten μ g of protein was separated on an SDS-12% polyacrylamide gel, transferred onto nitrocellulose membranes, and immunochemically stained with anti-HDAC2 and anti-HDAC3 antibodies (A). Protein cross-linked to DNA was incubated with or without alkaline phosphatase, separated on a SDS-12% polyacrylamide gel, and immunochemically stained with anti-HDAC2 antibodies (B).

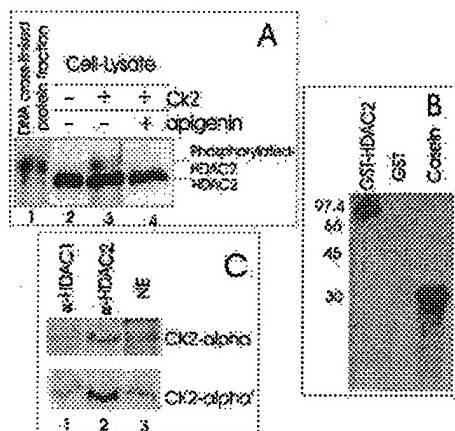


FIG. 4. Protein kinase CK2 phosphorylates HDAC2. An equal amount of cell lysate protein (20 μ g) was incubated with or without purified CK2 and with or without 80 μ M apigenin in the presence ATP. The control and treated samples and 10 μ g of protein cross-linked to DNA with cisplatin were loaded onto an SDS-12% polyacrylamide gel, transferred onto nitrocellulose membranes, and immunochemically stained with anti-HDAC2 antibody (A). Purified GST-HDAC2 fusion protein, GST, and casein were incubated with purified CK2 in the presence of [γ -³²P]ATP. The samples were loaded onto a SDS-15% polyacrylamide gel. The dried gel was autoradiographed (B). C, an equal amount of T5 cell lysate (2 A_{260}) was immunoprecipitated with 4 μ g of anti-HDAC1 and anti-HDAC2 antibodies. After washing with immunoprecipitation buffer, the beads were boiled in SDS loading buffer. The immunoprecipitated samples and nuclear extracted protein (10 μ g) were loaded onto an SDS-10% polyacrylamide gel, transferred to a nitrocellulose membrane, and immunochemically stained with anti-CK2 α and anti-CK2 α' antibodies.

was associated with HDAC2, HDAC2 and HDAC1 immunoprecipitates were analyzed by immunoblotting with anti-CK2 α or anti-CK2 α' antibodies (Fig. 4C). HDAC2 and to a lesser extent HDAC1 were bound to CK2. Immunoblotting experiments of Sp1 and Sp3 immunoprecipitates revealed the presence of CK2 (data not shown).

In immunoprecipitation and immunoblotting experiments we determined that most HDAC2 was in complex with HDAC1 in T5 breast cancer cells. HDAC1, which was more abundant than HDAC2, was in complex with HDAC2 and with other complexes not containing HDAC2. Incubation of HDAC1 and HDAC2 immunoprecipitates with alkaline phosphatase reduced the HDAC activity of the complexes (Fig. 5). The associated HDAC activities with Sp1 and Sp3 immunoprecipitates were also reduced when incubated with alkaline phosphatase.

In summary, we demonstrate that Sp3 and Sp1 are associated with HDAC1 and CK2-phosphorylated HDAC2. Although most HDAC2 is in an unmodified state, phosphorylated HDAC2 is preferentially associated with Sp1, Sp3, and chromatin in human breast cancer cells. CK2 is up-regulated in

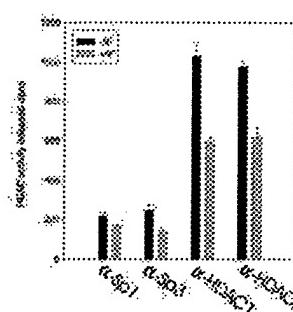


FIG. 5. Phosphatase digestion reduces HDAC activity associated with Sp1 and Sp3. An equal amount of cell lysate (2 A_{260}) was immunoprecipitated with anti-Sp1, anti-Sp3, anti-HDAC1, and anti-HDAC2 antibodies. These immunoprecipitated fractions were incubated with (+AP) or without (-AP) alkaline phosphatase, and HDAC activities were measured. Each value represents the mean \pm S.E. of three different preparations.

several cancers including breast cancer, and there is evidence that CK2 may promote breast cancer by deregulating key transcription processes (28–30). Many estrogen-induced genes (e.g. cathepsin D, c-fos, adenine deaminase, and c-myc) in human breast cancer cells have a half-site estrogen response element positioned next to a Sp1 binding site (31–33). Sp3 would compete with Sp1 to bind the regulatory regions of these genes. Both Sp1 and Sp3 may recruit HDAC1 and phosphorylated HDAC2 to these sites, whereas the estrogen receptor recruits histone acetyltransferases CBP (cAMP-response element-binding protein (CREB)-binding protein) and p300, resulting in dynamic acetylation of histones and transcription factors located at the promoters of these genes (4, 34, 35). CK2 phosphorylation of HDAC2 recruited by Sp1 or Sp3 would regulate HDAC activity and alter the balance of histone deacetylase and histone acetyltransferase activities and dynamic chromatin remodeling of these estrogen-regulated promoters.

REFERENCES

- Wang, X., He, C., Moore, S. C., and Ausio, J. (2001) *J. Biol. Chem.* **276**, 12764–12768
- Tse, C., Sera, T., Wolffe, A. P., and Hansen, J. C. (1998) *Mol. Cell. Biol.* **18**, 4629–4638
- Spencer, V. A., and Davie, J. R. (2001) *J. Biol. Chem.* **276**, 34810–34815
- Sun, J.-M., Chen, H. Y., and Davie, J. R. (2001) *J. Biol. Chem.* **276**, 49435–49442
- Davie, J. R., and Moniwa, M. (2000) *Crit Rev. Eukaryotic Gene Expression* **10**, 303–325
- Fischle, W., Kiermer, V., Dequiedt, F., and Verdin, E. (2001) *Biochem. Cell Biol.* **79**, 337–348
- Ferreira, R., Naguibneva, I., Pritchard, L. L., Ait-Si-Ali, S., and Harel-Bellan, A. (2001) *Oncogene* **20**, 3128–3133
- Lai, A., Kennedy, B. K., Barbie, D. A., Bertos, N. R., Yang, X. J., Theberge, M. C., Tsai, S. C., Seto, E., Zhang, Y., Kuzminich, A., Lane, W. S., Reinberg, D., Harlow, E., and Branton, P. E. (2001) *Mol. Cell. Biol.* **21**, 2918–2932
- Yao, Y. L., Yang, W. M., and Seto, E. (2001) *Mol. Cell. Biol.* **21**, 5979–5991
- Kennett, S. B., Uvdavia, A. J., and Horowitz, J. M. (1997) *Nucleic Acids Res.* **25**, 3110–3117
- Suske, G. (1999) *Gene (Amst.)* **238**, 291–300
- Gartel, A. L., Gouzman, E., Najmabadi, F., and Tyner, A. L. (2000) *Oncogene* **19**, 5182–5188
- Apt, D., Watts, R. M., Suske, G., and Bernard, H. U. (1996) *Virology* **224**, 281–291
- Yang, W. M., Yao, Y. L., Sun, J. M., Davie, J. R., and Seto, E. (1997) *J. Biol. Chem.* **272**, 28001–28007
- Samuel, S. K., Spencer, V. A., Bajno, L., Sun, J.-M., Holth, L. T., Oesterreich, S., and Davie, J. R. (1998) *Cancer Res.* **58**, 3004–3008
- Spencer, V. A., and Davie, J. R. (2002) in *The Protein Protocols Handbook* (Walker, J. M., ed) pp. 747–752, Humana Press, Totowa, NJ
- Spencer, V. A., and Davie, J. R. (2002) in *The Protein Protocols Handbook* (Walker, J. M., ed) pp. 753–760, Humana Press, Totowa, NJ
- Gietz, R. D., Graham, K. C., and Litchfield, D. W. (1995) *J. Biol. Chem.* **270**, 13017–13021
- Zhao, H., Hart, L. L., Keller, U., Holth, L. T., and Davie, J. R. (2002) *J. Cell. Biochem.* **86**, 365–375
- Hendzel, M. J., Delcave, G. P., and Davie, J. R. (1991) *J. Biol. Chem.* **266**, 21936–21942
- Sun, J.-M., Chen, H. Y., Litchfield, D. W., and Davie, J. R. (1996) *J. Cell. Biochem.* **62**, 454–466
- Mastrangelo, I. A., Courrey, A. J., Wall, J. S., Jackson, S. P., and Hough, P. V. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 5670–5674

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23. Pascal, E., and Tjian, R. (1991) *Genes Dev.* **5**, 1646–1656
24. Kennett, S. B., Moorefield, K. S., and Horowitz, J. M. (2002) *J. Biol. Chem.* **277**, 9780–9789
25. Doetzlhofer, A., Rotheneder, H., Lagger, G., Koranda, M., Kurtev, V., Brosch, G., Wintersberger, E., and Seiser, C. (1999) *Mol. Cell. Biol.* **19**, 5504–5511
26. Cai, R., Kwon, P., Yan-Neale, Y., Sambucetti, L., Fischer, D., and Cohen, D. (2001) *Biochem. Biophys. Res. Commun.* **283**, 445–453
27. Pflum, M. K., Tong, J. K., Lane, W. S., and Schreiber, S. L. (2001) *J. Biol. Chem.* **276**, 47733–47741
28. Landesman-Bollag, E., Romieu-Mourez, R., Song, D. H., Sonenschein, G. E., Cardiff, R. D., and Seldin, D. C. (2001) *Oncogene* **20**, 3247–3257
29. Landesman-Bollag, E., Song, D. H., Romieu-Mourez, R., Sussman, D. J., Cardiff, R. D., Sonenschein, G. E., and Seldin, D. C. (2001) *Mol. Cell Biochem.* **227**, 153–165
30. Tawfic, S., Yu, S., Wang, H., Faust, R., Davis, A., and Ahmed, K. (2001) *Histol. Histopathol.* **16**, 573–582
31. Safe, S. (2001) *Vitam. Horm.* **62**, 231–252
32. Dubik, D., and Shiu, R. P. (1992) *Oncogene* **7**, 1587–1594
33. Abdelrahim, M., Samudio, I., Smith, R., III, Burghardt, R., and Safe, S. (2002) *J. Biol. Chem.* **277**, 28815–28822
34. Chen, H., Lin, R. J., Xie, W., Wilpitz, D., and Evans, R. M. (1999) *Cell* **98**, 675–686
35. Shang, Y., Hu, X., DiRenzo, J., Lazar, M. A., and Brown, M. (2000) *Cell* **103**, 843–852

INHIBITION OF HISTONE DEACETYLASE ACTIVITY BY BUTYRATE

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Running title: Butyrate inhibition of histone deacetylases

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ABSTRACT

This article reviews the effect of the dietary micronutrient butyrate on histone deacetylase (HDAC) activity. Sodium butyrate has multiple effects on cultured mammalian cells, including inhibition of proliferation, induction of differentiation, and induction or repression of gene expression. The observation that butyrate treatment of cells resulted in histone hyperacetylation, initiated a flurry of activity leading to discoveries that butyrate inhibited HDAC activity. Butyrate has been an essential agent in determining the role of histone acetylation in chromatin structure and function. Interestingly, inhibition of HDAC activity affects the expression of only 2% of the mammalian genes. Promoters of butyrate responsive genes have butyrate response elements, with the action of butyrate often being mediated through Sp1/Sp3 binding sites (e.g., p21^{Waf1,Cip1}). We demonstrated that Sp3 and Sp1 recruit HDAC1 and HDAC2, with the later being phosphorylated by protein kinase CK2. A model is proposed in which inhibition of the Sp1/Sp3 associated HDAC activity leads to histone hyperacetylation and transcriptional activation of the p21^{Waf1,Cip1} gene. p21^{Waf1,Cip1} inhibits cyclin dependent kinase 2 activity, arresting cell cycling. Pending the cell background, the non-proliferating cells may enter differentiation or apoptotic pathways. The potential of butyrate and HDAC inhibitors in the prevention and treatment of cancer is presented.

Key Words: sodium butyrate, histone deacetylase, p21^{Waf1,Cip1}, histone acetylation, gene expression, Sp1, Sp3

Abbreviations used: HDAC, histone deacetylase; HAT, histone acetyltransferase, ER, estrogen receptor; AUT, acetic acid-urea-Triton X-100; Cdk, cyclin dependent kinase; Rb, retinoblastoma protein; HMG, high mobility group protein; TSA, trichostatin A.

INTRODUCTION

Butyrate is a short chain fatty acid produced by anaerobic bacterial fermentation of dietary fibers and resistant starches. It has been suggested that butyrate may inhibit the development of colon cancer (1,2). This article reviews the action of butyrate in altering gene expression and arresting cell proliferation by inhibiting the chromatin remodeling activity of histone deacetylases (HDACs).

MATERIALS AND METHODS

Cell culture. Human breast cancer cell line MCF-7 (T5) (ER positive and estrogen dependent) and MDA MB 231 (ER negative and estrogen independent) were cultured as described previously (3).

Pulse-chase labelling cells for analyses of histone acetylation rates. Human breast cancer cells and avian immature erythrocytes were pulse labelled with [³H]-acetate and then subsequently incubated in the absence of radiolabel and with sodium butyrate (10 mM) as described previously (3,4). Rates of histone acetylation were determined as previously described (5,6).

Immunoprecipitation. The following is an efficient method to solubilize nuclear proteins. MCF-7 (T5) human breast cancer cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl, pH8.0, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA) containing 1 mM PMSF, phosphatase inhibitors and protease inhibitors cocktail. The cells were sonicated twice for 15 sec. The cell lysate was collected by centrifugation at 10000 g for

10 minutes and incubated with anti-HDAC1, anti-HDAC2, anti-Sp1 or anti-Sp3 antibodies for 16 hours at 4°C (7).

Sequential immunoprecipitations. Sequential immunoprecipitations were done as described (7). Briefly, cell lysates were incubated with anti-Sp1 antibodies. The immunoprecipitated and immunodepleted (supernatant) fractions were collected. Secondary immunoprecipitations were done with the Sp1 immunodepleted (supernatant) fraction and with anti-Sp3 antibodies, and the immunoprecipitated and immunodepleted fractions collected.

RESULTS

Effect of butyrate on cell proliferation and HDACs. In the mid 1975s several research groups had reported that sodium butyrate halted DNA synthesis, arrested cell proliferation, altered cell morphology and increased or decreased the expression of genes (8). Treatment of erythroleukemic cells with butyrate was shown to be very effective in inducing differentiation in these cells (9). A turning point in understanding the mechanism of butyrate action was the observation by Ingram and colleagues that butyrate increased the level of acetylated histones in cultured HeLa and Friend erythroleukemic cells (10). Several chromatin groups interested in histone acetylation recognized that to increase histone acetylation either the activity of histone acetyltransferases (HATs) was increased or conversely the activity of HDACs was inhibited. The latter, inhibition of HDAC activity, was found to be the mode of butyrate action (11-14).

Histone acetylation, a dynamic process regulating chromatin structure.

HDACs catalyze the removal of acetate from modified lysine residues located in the N-terminal tail region of the core histones, H2A, H2B, H3 and H4 (Fig. 1A). These core histones form a histone octamer around which is wrapped 146 bp of DNA. The four core histones have a similar structure with a basic N terminal domain, a central histone fold domain, which mediates histone-histone and histone-DNA interactions, and a C terminal tail (15). The crystal structure of the nucleosome shows that the N terminal tails emanate from the nucleosome in all directions (Fig. 1B) (16). Reversible acetylation occurs on specific lysines located in the N terminal tail domains of the core histones (Fig. 1A). With the exception of H2A, the core histones are acetylated at four to five sites. Thus, a nucleosome has potentially 28 or more sites of acetylation. In addition to acetylation, the core histones are modified by methylation, phosphorylation, and ubiquitination (17).

Although we have known since the 1960s that histone acetylation has a role in chromatin structure and function, we still know little about what this modification is doing to remodel chromatin structure (18). However, one function of histone acetylation is altering the compaction of chromatin. Acetylation of the histone tails disrupts higher order chromatin folding (19) and promotes the solubility of chromatin at physiological ionic strength (20). Nucleosomes do not have to be maximally acetylated to prevent chromatin compaction. Hansen and colleagues demonstrated that acetylation to 46% of maximal site occupancy was sufficient to prevent higher order folding and stimulation of transcription by RNA polymerase III (21). It has been proposed that acetylation of core histone tails interferes with interactions with proteins and/or DNA, thereby destabilizing higher order chromatin organization (22,23). These combined effects of histone

acetylation on the destabilization of chromatin structure facilitate transcription (21,24) (Fig. 1C).

Enzymes catalyzing dynamic histone acetylation. The steady state of acetylated histones in a eukaryotic cell and at a specific gene locus is governed by the net activities of HATs and HDACs (Fig. 2). HATs often have transcriptional coactivator activity and when recruited to a gene promoter by a transcription factor will increase the level of acetylated histones and enhance transcriptional activity of the promoter (17,25). The most potent HATs in mammalian cells are CBP, p300, PCAF and Tip60 (17,26). SRC-1 and SRC-3 are HATs recruited by steroid receptors (17).

Three classes of HDACs are known. Class I HDACs consist of mammalian HDACs, HDAC1, HDAC2 (the mammalian homologue of yeast RPD3), HDAC3 and HDAC8. Class 2 HDACs include mammalian HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10 (27-32). Class 3 HDACs are members of the sirtuin (SIRT) family of HDACs, among which yeast Sir2 is the founding member (30).

Butyrate inhibits most HDACs except class 3 HDACs and class 2 HDACs 6 and 10. In inhibiting HDAC activity HAT activity continues, resulting in histone hyperacetylation. Histones, however, are not the only substrates of these enzymes. HMG proteins are acetylated. This modification has a wide range of affects on function of the HMG in remodeling chromatin structure and in regulating gene expression (33-35). Multiple transcription factors are acetylated (36) (Fig. 2). Acetylation of a transcription factor may alter its properties (37). For example, CBP acetylates p53 and GATA-1 and potentiates the activities of these transcription factors (36,38).

Dynamic histone acetylation – rates of acetylation and deacetylation. Histone acetylation is a dynamic process, occurring at different rates. In mammalian cells, one population of core histones is characterized by rapid hyperacetylation and rapid deacetylation ($t\frac{1}{2} = 3$ to 7 min). This highly dynamic acetylation-deacetylation is limited to 10 to 15% of the core histones (3). A second population is acetylated and deacetylated at a slower rate ($t\frac{1}{2} = 30$ min) (39). Approximately 60 to 70% of the histones of cultured mammalian cells participate in reversible acetylation. The remainder of the histones is "frozen" in low or non-acetylated states (25).

Incubation of human breast cancer cells (MDA-MB-231) cells with sodium butyrate for 2 hours has a major impact on the steady state levels of acetylated histones (see acetylated H4 levels in Fig. 3). Histones were electrophoretically resolved on an AUT 15% polyacrylamide gel, which resolve histones according to their size, charge and hydrophobicity. Thus this gel system is ideal for separating modified histones and histone variants. For example, H3 has three variants H3.1, H3.2 and H3.3 which are resolved with this gel system (40).

To study the fast rate of histone acetylation, cells were pulse labeled with [3 H] acetate for 15 min, the label removed and then the cells incubated with sodium butyrate to drive the dynamically acetylated histones into highly acetylated isoforms (Fig. 3, fluorogram). The fluorogram clearly shows the movement of the label in H4, H2B and H3 moving into the highly acetylated isoforms.

In contrast to mammalian cells in culture, only 2% of the histones in the terminally differentiated avian immature erythrocyte participate in dynamic acetylation. Further only the fast rate of histone acetylation is observed in these cells (6). Thus a shift in the steady state of acetylated histone is not observed on the stained AUT gel pattern when

avian cells are incubated with sodium butyrate for one hour. Pulse labeling of the cells with [³H] acetate for 15 min followed by a chase for 60 min in the presence of butyrate rapidly drives the histones participating in dynamic acetylation into the highest acetylated isoforms. The dynamically acetylated histones are limited to transcriptionally active and competent regions of the avian erythrocyte genome (41). In mammalian cells the bulk of the dynamically acetylated histone may serve a surveillance function (42).

By measuring the rate of loss of label in the monoacetylated histone isoform (e.g., H4, H2B, H3.2 in Fig. 3), the rate of histone acetylation is determined. Fig. 4 shows that H4, H3.2 and H2B have two rates of acetylation in human breast cancer cells (MCF-7 (T5)). For H4 the fast rate of acetylation has a $t\frac{1}{2} = 8$ minutes, while the slower rate of acetylation has a $t\frac{1}{2} = 200$ to 350 minutes (3,39).

Butyrate – mode of action. Recently the crystal structure of a HDAC like protein (HDLP) from the hyperthermophilic bacterium *Aquifex aeolicus* with the HDAC inhibitor TSA was reported (43) (Fig. 5). The structure shows the position of the essential zinc atom involved in catalysis of class 1 and 2 HDACs. HDLP shares 35.2% similarity over a 390 residue region with mammalian HDAC1; this region constitutes the deacetylase core. The aliphatic chain of TSA occupies a hydrophobic cleft on the surface of the enzyme (Fig. 5). Possibly two butyrate molecules could also occupy the hydrophobic pocket and inhibit the enzyme. However, butyrate was found to a non-competitive inhibitor of HDAC, arguing that butyrate does not associate with the substrate binding site (44). The binding site and mechanism by which butyrate inhibits HDAC activity remain unanswered questions.

HDAC complexes and transcription. Mammalian HDAC1 and HDAC2 are in large multiprotein complexes, Sin3 and NuRD (Fig. 6). The Sin3 complex, which has been estimated size of 1-2 MDa, contains mSin3, SAP18, SAP30, and retinoblastoma associated proteins (RbAp) 46 and 48 (25,30,45,46). The Sin3 complex is directed to its target chromatin location by sequence specific DNA binding proteins that interact directly with mSin3 and other components of Sin3 complex. Some examples of DNA binding proteins that recruit the Sin3 complex include the Mad-family proteins, unliganded hormone receptors, MeCP2, p53, REST, and the Ikaros-family proteins (25,30,46,47).

Another complex called NuRD (nucleosome remodelling histone deacetylase complex) is about 2 MDa in size and consists of MTA2 (highly related to metastasis-associated protein MTA1), Mi2, RbAp46/48 and MBD3 (methyl-CpG-binding domain-containing protein). NuRD has both ATP-dependent chromatin remodeling and HDAC activities (25,30).

When ER is bound to hydroxytamoxifen, ER will recruit NCoR/SMRT and HDACs to the promoter of an estrogen responsive promoter, repressing promoter activity. However, when estradiol is bound to the ER, ER will recruit coactivator/HATs and chromatin remodeling complexes to the promoter, enabling transcription (48,49). It is important to note that the steady state level of acetylation at a regulatory element (e.g., promoter) or along the coding region of a gene will be dictated by the balance of HATs and HDACs recruited to those sites. As histone acetylation on-going at transcriptionally active genes is a dynamic and rapid process, alterations in recruitment of factors for HATs or HDACs will quickly change the balance of these two activities towards increasing or decreasing the steady state level of acetylated histones. For example, a

ligand binding steroid receptor or a phosphorylated transcription factor (e.g., NF- κ B) can be quickly changed to recruiting HDACs to HATs and vice versa (50,51).

Effect of estradiol on global histone acetylation dynamics in human breast cancer cells. The following study is an example of studies using sodium butyrate to determine histone acetylation and deacetylation rates. In this study, we investigated the effect of estradiol on global dynamic histone acetylation in hormone responsive human breast cancer cells (3). Histone acetylation labeling experiments and immunoblot analyses of dynamically acetylated histones show that estradiol rapidly increases histone acetylation in ER positive, hormone dependent MCF-7 (T5) human breast cancer cells. The effect of estradiol on the rates of histone acetylation and deacetylation in MCF-7 (T5) cells was determined. Estradiol increased the level of acetylated histones by reducing the rate of histone deacetylation, while the rates of histone acetylation were not altered.

Butyrate response element and gene expression. Studies have revealed that among the fatty acids, butyrate is the most effective in inhibiting HDAC activity and in arresting cell proliferation (52). Butyrate was also the most effective fatty acid in stimulating or repressing the expression of specific genes (Table 1). Considering butyrate's action to inhibit HDAC activity and to promote histone hyperacetylation (see Fig. 3), it was surprising to learn that expression of only 2% of the mammalian genes was affected when HDAC activity was inhibited (53,54).

Within the promoter of butyrate responsive genes is found a butyrate response element (55-59). It appears that these butyrate elements may be put into different

groups depending on the DNA sequence of the butyrate response element (**Table 2**). One group of genes that are either induced or repressed by butyrate have a common DNA sequence in their butyrate response elements, suggesting that a common transcription factor binds to this site. Another group, which includes the Cdk2 inhibitor, p21^{Waf1,Cip1}, shares a Sp1/Sp3 binding site(s) in their butyrate response elements.

Sp1, Sp3 and recruitment of HDAC. Sp1 and Sp3 are ubiquitously expressed mammalian transcription factors that function as activators or repressors. Sp1 and Sp3 bind with equivalent affinity to GC-boxes through their three zinc fingers located in the C terminal region of the protein (60). Activation domains A and B (Gln-rich and S/T-rich regions) are located in the N-terminal part of the protein, while the D domain, which is found in the C-terminal region, is involved in multimerization (61,62). Synergistic transcriptional activation is mediated through the capacity of Sp1's D domain to form multimers (61,62). Scanning transmission electron microscopy provided evidence that Sp1 first forms a tetramer and then assembles multiple stacked tetramers at the DNA binding site (61). The interesting feature of this structure is that a Sp1 multimer would present several interacting surfaces to proteins associating with Sp1 (e.g., p300/CBP, HDAC1, TAF_{II} subunits of TFIID, CRSP, E2F1, and ER (63-66)). The net activity of these factors to promote or hinder transcription would depend on the abundance, affinity and residence time of these factors on the Sp1 multimer.

Sp3 has three isoforms, a long (L-Sp3) and two short forms (M1-Sp3, M2-Sp3) that are the products of differential translational initiation (67,68). The protein structure of L-Sp3 is very similar to that of Sp1, except that Sp3 has a repression domain located

N-terminal to the zinc finger DNA-binding domain (60). The factors regulating the translational initiation of Sp3 mRNA are currently not known.

Although Sp1 and Sp3 share a common D domain involved in forming multimers, we reported that Sp1 and Sp3 form separate complexes in estrogen dependent human breast cancer cells (7). In performing these studies we wanted to ensure efficient solubilization of nuclear proteins as Sp1 and Sp3 are tightly bound to the nucleus of MCF-7 (T5) breast cancer cells (see Materials and Methods). Sequential immunoprecipitations were done, first with anti-Sp1 antibodies and then with anti-Sp3 antibodies (see Materials and Methods). Fig. 7 shows that Sp1 and Sp3 form separate complexes.

Next we determined whether Sp1 and Sp3 were associated with HDAC activity. A previous report had shown that Sp1 was associated with HDAC1 (64). Both Sp1 and Sp3 were associated with HDAC activity in human breast cancer cells (7). In immunoblot analyses of the Sp1 and Sp3 immunoprecipitated complexes we observed that HDAC1, HDAC2 but not HDAC3 were associated with Sp1 and Sp3 (Fig. 8). However, it was very interesting to find a major enrichment of a slower migrating HDAC2 species associating with Sp1 and Sp3. Further investigation revealed that this slower migrating species was protein kinase CK2 phosphorylated HDAC2 (7,69). Alkaline phosphatase treatment of HDAC2, Sp1 and Sp3 complexes reduced the associated HDAC activity.

Protein kinase CK2 is a tetramer consisting of two alpha (or alpha prime) and two beta subunits (70). In immunoprecipitation experiments, we found that CK2 was associated with HDAC2 and to lesser levels with HDAC1 (Fig. 9). Although we find that HDAC2 is associated with MBD3, a component of the NuRD HDAC complex, and with

Sin3A, a component of Sin3 complex (Fig. 6), it remains to be determined if CK2 or HDAC2 is associated with either of these complexes. CK2 is upregulated in several cancers including breast cancer, and there is evidence that CK2 may promote breast cancer by deregulating key transcription processes (71-74).

A model for the butyrate induction of p21^{Waf1,Cip1} gene expression and inhibition of cell cycle. The p21^{Waf1,Cip1} promoter has six Sp1 binding sites (the butyrate response element). Evidence has been presented that Sp3 and not Sp1 is associated with this promoter (75). Further, a Sp1 like protein ZBP-89 is associated with one or more of the Sp1 sites. ZBP-89 recruits p300, a coactivator/HAT (76). Thus ZBP-89 would recruit HAT activity to the promoter, while Sp3 would recruit HDAC activity to the p21^{Waf1,Cip1} promoter, resulting in dynamic histone acetylation (Fig. 10). The steady state level of acetylated histones associated with the p21^{Waf1,Cip1} promoter is low, favoring a condensed chromatin structure and inactive promoter (77). Inhibition of the HDAC activity with sodium butyrate would allow the HAT activity of p300 to increase the histone acetylation levels at the promoter and nearby regions (77). Hyperacetylation of the histones would support chromatin opening and induction of p21^{Waf1,Cip1} gene expression.

In the transition from G1 to S phase of the cell cycle, p21^{Waf1,Cip1} has a key role (Fig. 11). Initially there is an increase in p21^{Waf1,Cip1} expression following the transient activation of the ERKs and the Ras-mitogen activated protein kinase pathway (78). p21^{Waf1,Cip1} will inhibit the activity of cyclin E-Cdk2 kinase and promote the assembly of stable cyclin D1-Cdk4/6 kinase complexes (79). Subsequently, p21^{Waf1,Cip1} gene expression is repressed, resulting in the lowering of p21^{Waf1,Cip1} protein levels and the

activation of cyclin E-Cdk2. Cyclin E-Cdk2 activity is required for the final stage phosphorylation of Rb and the release of the transcription factor E2F, which will induce the expression of genes involved in taking cells through S phase (DNA synthesis phase) of the cell cycle (25,79-81). Butyrate will induce expression of p21^{Waf1,Cip1}, inhibiting cyclin E-Cdk2 activity and halting the subsequent events required for cells to enter S phase. The cell cycle arrested cells may differentiate or undergo cell death by apoptosis.

Butyrate and HDAC inhibitors in the prevention and treatment of cancer.

By inhibiting the HDAC activity recruited to the p21^{Waf1,Cip1} promoter by Sp1 or Sp3, butyrate induces the expression of p21^{Waf1,Cip1}, stopping cell proliferation. This is a p53 independent process (82). Several studies suggest that the production of butyrate in the colon may be protective against colon carcinogenesis (1,2). Current studies and clinical trials strongly suggest that HDAC inhibitors, such as TSA and SAHA, which also induce p21^{Waf1,Cip1} expression, will be effective in arresting cancer cell proliferation, leading to cell's differentiating as in acute promelocytic anemia or undergoing apoptosis (83-88). These new strategies to prevention and treatment of cancer have been termed as "gene-regulating chemoprevention", "gene-regulating chemotherapy" and "transcription therapy" (88,89). No matter which term wins the day, these are exciting times for the dietary micronutrient, butyrate, and HDAC inhibitors in the challenge of preventing and treating cancer.

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LITERATURE CITED

1. Hinnebusch, B. F., Meng, S., Wu, J. T., Archer, S. Y. & Hodin, R. A. (2002) The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. *J.Nutr.* 132: 1012-1017.
2. Emenaker, N. J., Calaf, G. M., Cox, D., Basson, M. D. & Qureshi, N. (2001) Short-chain fatty acids inhibit invasive human colon cancer by modulating uPA, TIMP-1, TIMP-2, mutant p53, Bcl-2, Bax, p21 and PCNA protein expression in an in vitro cell culture model. *J.Nutr.* 131: 3041S-3046S.
3. Sun, J.-M., Chen, H. Y. & Davie, J. R. (2001) Effect of estradiol on histone acetylation dynamics in human breast cancer cells. *J.Biol.Chem.* 276: 49435-49442.
4. Hendzel, M. J., Delcuve, G. P. & Davie, J. R. (1991) Histone deacetylase is a component of the internal nuclear matrix. *J.Biol.Chem.* 266: 21936-21942.
5. Covault, J. & Chalkley, R. (1980) The identification of distinct populations of acetylated histone. *J.Biol.Chem.* 255: 9110-9116.
6. Zhang, D.-E. & Nelson, D. A. (1988) Histone acetylation in chicken erythrocytes: rates of acetylation and evidence that histones in both active and potentially active chromatin are rapidly modified. *Biochem.J.* 250: 233-240.
7. Sun, J. M., Chen, H. Y., Moniwa, M., Litchfield, D. W., Seto, E. & Davie, J. R. (2002) The transcriptional repressor Sp3 is associated with CK2 phosphorylated histone deacetylase 2. *J.Biol.Chem.* 277: 35783-35786.
8. Prasad, K. N. & Sinha, P. K. (1976) Effect of sodium butyrate on mammalian cells in culture: a review. *In Vitro* 12: 125-132.
9. Leder, A. & Leder, P. (1975) Butyric acid, a potent inducer of erythroid differentiation in cultured erythroleukemic cells. *Cell* 5: 319-322.
10. Riggs, M. G., Whittaker, R. G., Neumann, J. R. & Ingram, V. M. (1977) n-Butyrate causes histone modification in HeLa and Friend erythroleukaemia cells. *Nature* 268: 462-464.
11. Candido, E. P. M., Reeves, R. & Davie, J. R. (1978) Sodium butyrate inhibits histone deacetylation in cultured cells. *Cell* 14: 105-113.
12. Sealy, L. & Chalkley, R. (1978) The effect of sodium butyrate on histone modification. *Cell* 14: 115-121.
13. Boffa, L. C., Vidali, G., Mann, R. S. & Allfrey, V. G. (1978) Suppression of histone deacetylation *in vivo* and *in vitro* by sodium butyrate. *J.Biol.Chem.* 253: 3364-3366.

14. Vidali, G., Boffa, L. C., Bradbury, E. M. & Allfrey, V. G. (1978) Suppression of histone deacetylation by butyrate leads to accumulation of multi-acetylated forms of histones H3 and H4 and increased DNase I-sensitivity of the associated DNA sequences. *Proc.Natl.Acad.Sci.U.S.A.* 75: 2239-2243.
15. Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F. & Richmond, T. J. (1997) Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* 389: 251-260.
16. Luger, K. & Richmond, T. J. (1998) The histone tails of the nucleosome. *Curr.Opin.Genet.Dev.* 8: 140-146.
17. Spencer, V. A. & Davie, J. R. (1999) Role of covalent modifications of histones in regulating gene expression. *Gene* 240: 1-12.
18. Pogo, B. G., Allfrey, V. G. & Mirsky, A. E. (1966) RNA synthesis and histone acetylation during the course of gene activation in lymphocytes. *Proc.Natl.Acad.Sci.U.S.A.* 55: 805-812.
19. Garcia-Ramirez, M., Rocchini, C. & Ausio, J. (1995) Modulation of chromatin folding by histone acetylation. *J.Biol.Chem.* 270: 17923-17928.
20. Wang, X., He, C., Moore, S. C. & Ausio, J. (2001) Effects of histone acetylation on the solubility and folding of the chromatin fiber. *J.Biol.Chem.* 276: 12764-12768.
21. Tse, C., Sera, T., Wolffe, A. P. & Hansen, J. C. (1998) Disruption of higher order folding by core histone acetylation dramatically enhances transcription of nucleosomal arrays by RNA polymerase III. *Mol.Cell.Biol.* 18: 4629-4638.
22. Hansen, J. C., Tse, C. & Wolffe, A. P. (1998) Structure and function of the core histone N-termini: more than meets the eye. *Biochemistry* 37: 17637-17641.
23. Wang, X., Moore, S. C., Laszczak, M. & Ausio, J. (2000) Acetylation increases the alpha-helical content of the histone tails of the nucleosome. *J.Biol.Chem.*
24. Nightingale, K. P., Wellinger, R. E., Sogo, J. M. & Becker, P. B. (1998) Histone acetylation facilitates RNA polymerase II transcription of the drosophila hsp26 gene in chromatin. *EMBO J.* 17: 2865-2876.
25. Davie, J. R. & Moniwa, M. (2000) Control of chromatin remodeling. *Crit Rev.Eukaryot.Gene Expr.* 10: 303-325.
26. Stern, D. E. & Berger, S. L. (2000) Acetylation of histones and transcription-related factors. *Microbiol.Mol.Biol.Rev.* 64: 435-459.
27. Fischle, W., Emiliani, S., Hendzel, M. J., Nagase, T., Nomura, N., Voelter, W. & Verdin, E. (1999) A new family of human histone deacetylases related to *Saccharomyces cerevisiae* HDA1p. *J.Biol.Chem.* 274: 11713-11720.

28. Grozinger, C. M., Hassig, C. A. & Schreiber, S. L. (1999) Three proteins define a class of human histone deacetylases related to yeast Hda1p. *Proc.Natl.Acad.Sci.U.S.A.* 96: 4868-4873.
29. Fischle, W., Kiermer, V., Dequiedt, F. & Verdin, E. (2001) The emerging role of class II histone deacetylases. *Biochem.Cell Biol.* 79: 337-348.
30. Grozinger, C. M. & Schreiber, S. L. (2002) Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. *Chem.Biol.* 9: 3-16.
31. Guardiola, A. R. & Yao, T. P. (2002) Molecular cloning and characterization of a novel histone deacetylase HDAC10. *J.Biol.Chem.* 277: 3350-3356.
32. Kao, H. Y., Lee, C. H., Komarov, A., Han, C. C. & Evans, R. M. (2002) Isolation and characterization of mammalian HDAC10, a novel histone deacetylase. *J.Biol.Chem.* 277: 187-193.
33. Herrera, J. E., Sakaguchi, K., Bergel, M., Trieschmann, L., Nakatani, Y. & Bustin, M. (1999) Specific acetylation of chromosomal protein HMG-17 by PCAF alters its interaction with nucleosomes. *Mol.Cell Biol.* 19: 3466-3473.
34. Munshi, N., Merika, M., Yie, J., Senger, K., Chen, G. & Thanos, D. (1998) Acetylation of HMG I(Y) by CBP turns off IFN beta expression by disrupting the enhanceosome. *Mol.Cell* 2: 457-467.
35. Sternen, R., Vidali, G. & Allfrey, V. G. (1979) Studies of acetylation and deacetylation in high mobility group proteins: identification of the sites of acetylation in HMG 1. *J.Biol.Chem.* 254: 11577-11583.
36. Braun, H., Koop, R., Ertmer, A., Nacht, S. & Suske, G. (2001) Transcription factor Sp3 is regulated by acetylation. *Nucleic Acids Res.* 29: 4994-5000.
37. Cheung, W. L., Briggs, S. D. & Allis, C. D. (2000) Acetylation and chromosomal functions. *Curr.Opin.Cell Biol.* 12: 326-333.
38. Berger, S. L. (1999) Gene activation by histone and factor acetyltransferases. *Curr.Opin.Cell Biol.* 11: 336-341.
39. Davie, J. R. (1997) Nuclear matrix, dynamic histone acetylation and transcriptionally active chromatin. *Mol.Biol.Rep.* 24: 197-207.
40. Wu, R. S., Panusz, H. T., Hatch, C. L. & Bonner, W. M. (1986) Histones and their modifications. *CRC Crit.Rev.Biochem.* 20: 201-263.
41. Spencer, V. A. & Davie, J. R. (2001) Dynamically acetylated histones association with transcriptionally active and competent genes in the avian adult β -globin gene domain. *J.Biol.Chem.* 276: 34810-34815.

42. Perry, M. & Chalkley, R. (1982) Histone acetylation increases the solubility of chromatin and occurs sequentially over most of the chromatin. A novel model for the biological role of histone acetylation. *J.Biol.Chem.* 257: 7336-7347.
43. Finnin, M. S., Donigian, J. R., Cohen, A., Richon, V. M., Rifkind, R. A., Marks, P. A., Breslow, R. & Pavletich, N. P. (1999) Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature* 401: 188-193.
44. Cousins, L. S., Gallwitz, D. & Alberts, B. M. (1979) Different accessibilities in chromatin to histone acetylase. *J.Biol.Chem.* 254: 1716-1723.
45. Ayer, D. E. (1999) Histone deacetylases: transcriptional repression with SINers and NuRDs. *Trends.Cell Biol.* 9: 193-198.
46. Knoepfler, P. S. & Eisenman, R. N. (1999) Sin meets NuRD and other tails of repression. *Cell* 99: 447-450.
47. Gray, S. G. & Teh, B. T. (2001) Histone acetylation/deacetylation and cancer: an "open" and "shut" case? *Curr.Mol.Med.* 1: 401-429.
48. Shang, Y., Hu, X., DiRenzo, J., Lazar, M. A. & Brown, M. (2000) Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell* 103: 843-852.
49. Hart, L. L. & Davie, J. R. (2002) The estrogen receptor: more than the average transcription factor. *Biochem.Cell Biol.* 80: 335-341.
50. Ghosh, S. (1999) Regulation of inducible gene expression by the transcription factor NF-kappaB. *Immunol.Res.* 19: 183-189.
51. Zhong, H., May, M. J., Jimi, E. & Ghosh, S. (2002) The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. *Mol.Cell* 9: 625-636.
52. Kruh, J. (1982) Effects of sodium butyrate, a new pharmacological agent, on cells in culture. *Mol.Cell Biochem.* 42: 65-82.
53. Van Lint, C., Emiliani, S. & Verdin, E. (1996) The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. *Gene Expr.* 5: 245-253.
54. Mariadason, J. M., Corner, G. A. & Augenlicht, L. H. (2000) Genetic reprogramming in pathways of colonic cell maturation induced by short chain fatty acids: comparison with trichostatin A, sulindac, and curcumin and implications for chemoprevention of colon cancer. *Cancer Res.* 60: 4561-4572.
55. Yang, J., Kawai, Y., Hanson, R. W. & Arinze, I. J. (2001) Sodium butyrate induces transcription from the G alpha(i2) gene promoter through multiple Sp1 sites in

the promoter and by activating the MEK-ERK signal transduction pathway. *J.Biol.Chem.* 276: 25742-25752.

56. Siavoshian, S., Segain, J. P., Kornprobst, M., Bonnet, C., Cherbut, C., Galmiche, J. P. & Blottiere, H. M. (2000) Butyrate and trichostatin A effects on the proliferation/differentiation of human intestinal epithelial cells: induction of cyclin D3 and p21 expression. *Gut* 46: 507-514.

57. Tran, C. P., Familiari, M., Parker, L. M., Whitehead, R. H. & Giraud, A. S. (1998) Short-chain fatty acids inhibit intestinal trefoil factor gene expression in colon cancer cells. *Am.J.Physiol* 275: G85-G94.

58. Lu, Y. & Lotan, R. (1999) Transcriptional regulation by butyrate of mouse galectin-1 gene in embryonal carcinoma cells. *Biochim.Biophys.Acta* 1444: 85-91.

59. Walker, G. E., Wilson, E. M., Powell, D. & Oh, Y. (2001) Butyrate, a histone deacetylase inhibitor, activates the human IGF binding protein-3 promoter in breast cancer cells: molecular mechanism involves an Sp1/Sp3 multiprotein complex. *Endocrinology* 142: 3817-3827.

60. Suske, G. (1999) The Sp-family of transcription factors. *Gene* 238: 291-300.

61. Mastrangelo, I. A., Courey, A. J., Wall, J. S., Jackson, S. P. & Hough, P. V. (1991) DNA looping and Sp1 multimer links: a mechanism for transcriptional synergism and enhancement. *Proc.Natl.Acad.Sci.U.S.A.* 88: 5670-5674.

62. Pascal, E. & Tjian, R. (1991) Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. *Genes Dev.* 5: 1646-1656.

63. Ryu, S., Zhou, S., Ladurner, A. G. & Tjian, R. (1999) The transcriptional cofactor complex CRSP is required for activity of the enhancer-binding protein Sp1. *Nature* 397: 446-450.

64. Doetzlhofer, A., Rotheneder, H., Lagger, G., Koranda, M., Kurtev, V., Brosch, G., Wintersberger, E. & Seiser, C. (1999) Histone deacetylase 1 can repress transcription by binding to Sp1. *Mol.Cell Biol.* 19: 5504-5511.

65. Xiao, H., Hasegawa, T. & Isobe, K. (2000) p300 collaborates with Sp1 and Sp3 in p21(waf1/cip1) promoter activation induced by histone deacetylase inhibitor. *J.Biol.Chem.* 275: 1371-1376.

66. Porter, W., Saville, B., Hoivik, D. & Safe, S. (1997) Functional synergy between the transcription factor Sp1 and the estrogen receptor. *Mol.Endocrinol.* 11: 1569-1580.

67. Kennett, S. B., Udvadia, A. J. & Horowitz, J. M. (1997) Sp3 encodes multiple proteins that differ in their capacity to stimulate or repress transcription. *Nucleic Acids Res.* 25: 3110-3117.

68. Philipsen, S. & Suske, G. (1999) A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. *Nucleic Acids Res.* 27: 2991-3000.
69. Tsai, S. C. & Seto, E. (2002) Regulation of histone deacetylase 2 by protein kinase CK2. *J.Biol.Chem.* 277: 31826-31833.
70. Niefeld, K., Guerra, B., Ermakowa, I. & Issinger, O. G. (2001) Crystal structure of human protein kinase CK2: insights into basic properties of the CK2 holoenzyme. *EMBO J.* 20: 5320-5331.
71. Landesman-Bollag, E., Song, D. H., Romieu-Mourez, R., Sussman, D. J., Cardiff, R. D., Sonenshein, G. E. & Seldin, D. C. (2001) Protein kinase CK2: signaling and tumorigenesis in the mammary gland. *Mol.Cell Biochem.* 227: 153-165.
72. Landesman-Bollag, E., Romieu-Mourez, R., Song, D. H., Sonenshein, G. E., Cardiff, R. D. & Seldin, D. C. (2001) Protein kinase CK2 in mammary gland tumorigenesis. *Oncogene* 20: 3247-3257.
73. Tawfic, S., Yu, S., Wang, H., Faust, R., Davis, A. & Ahmed, K. (2001) Protein kinase CK2 signal in neoplasia. *Histol.Histopathol.* 16: 573-582.
74. Pflum, M. K., Tong, J. K., Lane, W. S. & Schreiber, S. L. (2001) Histone deacetylase 1 phosphorylation promotes enzymatic activity and complex formation. *J.Biol.Chem.* 276: 47733-47741.
75. Sowa, Y., Orita, T., Minamikawa-Hiranabe, S., Mizuno, T., Nomura, H. & Sakai, T. (1999) Sp3, but not Sp1, mediates the transcriptional activation of the p21/WAF1/Cip1 gene promoter by histone deacetylase inhibitor. *Cancer Res.* 59: 4266-4270.
76. Bai, L. & Merchant, J. L. (2000) Transcription factor ZBP-89 cooperates with histone acetyltransferase p300 during butyrate activation of p21waf1 transcription in human cells. *J.Biol.Chem.* 275: 30725-30733.
77. Richon, V. M., Sandhoff, T. W., Rifkind, R. A. & Marks, P. A. (2000) Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. *Proc.Natl.Acad.Sci.U.S.A.*
78. Bottazzi, M. E., Zhu, X., Bohmer, R. M. & Assoian, R. K. (1999) Regulation of p21(cip1) expression by growth factors and the extracellular matrix reveals a role for transient ERK activity in G1 phase. *J.Cell Biol.* 146: 1255-1264.
79. Cheng, M., Olivier, P., Diehl, J. A., Fero, M., Roussel, M. F., Roberts, J. M. & Sherr, C. J. (1999) The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. *EMBO J.* 18: 1571-1583.
80. Assoian, R. K. (1997) Anchorage-dependent cell cycle progression. *J.Cell Biol.* 136: 1-4.

81. Roovers, K. & Assoian, R. K. (2000) Integrating the MAP kinase signal into the G1 phase cell cycle machinery. *BioEssays* 22: 818-826.
82. Nakano, K., Mizuno, T., Sowa, Y., Orita, T., Yoshino, T., Okuyama, Y., Fujita, T., Ohtani-Fujita, N., Matsukawa, Y., Tokino, T., Yamagishi, H., Oka, T., Nomura, H. & Sakai, T. (1997) Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. *J.Biol.Chem.* 272: 22199-22206.
83. Butler, L. M., Webb, Y., Agus, D. B., Higgins, B., Tolentino, T. R., Kutko, M. C., LaQuaglia, M. P., Drobnyak, M., Cordon-Cardo, C., Scher, H. I., Breslow, R., Richon, V. M., Rifkind, R. A. & Marks, P. A. (2001) Inhibition of transformed cell growth and induction of cellular differentiation by pyroxamide, an inhibitor of histone deacetylase. *Clin.Cancer Res.* 7: 962-970.
84. Marks, P. A., Rifkind, R. A., Richon, V. M. & Breslow, R. (2001) Inhibitors of histone deacetylase are potentially effective anticancer agents. *Clin.Cancer Res.* 7: 759-760.
85. Richon, V. M., Zhou, X., Rifkind, R. A. & Marks, P. A. (2001) Histone deacetylase inhibitors: development of suberoylanilide hydroxamic acid (SAHA) for the treatment of cancers. *Blood Cells Mol.Dis.* 27: 260-264.
86. Marks, P. A., Richon, V. M. & Rifkind, R. A. (2000) Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. *J.Natl.Cancer Inst.* 92: 1210-1216.
87. Warrell, R. P., Jr., He, L. Z., Richon, V., Calleja, E. & Pandolfi, P. P. (1998) Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. *J.Natl.Cancer Inst.* 90: 1621-1625.
88. He, L. Z., Tolentino, T., Grayson, P., Zhong, S., Warrell, R. P., Jr., Rifkind, R. A., Marks, P. A., Richon, V. M. & Pandolfi, P. P. (2001) Histone deacetylase inhibitors induce remission in transgenic models of therapy-resistant acute promyelocytic leukemia. *J.Clin.Invest* 108: 1321-1330.
89. Sowa, Y. & Sakai, T. (2000) Butyrate as a model for "gene-regulating chemoprevention and chemotherapy.". *Biofactors* 12: 283-287.

TABLE 1*Effects of fatty acids on mammalian cells in culture (52)*

No. carbons in fatty acid	Effect on fibroblast growth (% control)	Induction of alkaline phosphatase (HeLa) (% control)	Inhibition of estradiol induced synthesis of transferrin mRNA	Inhibition of HDAC (calf thymus)
C2 (acetate)	82	170	18	10
C3 (propionate)	45	160	77	60
C4 (butyrate)	0	630	95	80
C5 (valeroic)	71	420		65
C6 (caproate)		120		30

TABLE 2
Butyrate response elements (55-59)

Gene and butyrate response	Butyrate Response Element
Group 1	
Cyclin D1 (repression)	AGCCACCTCCA
Intestinal trefoil factor (repression) AG
Calbindin-D28k (induction) A.G
Metallothionein IIA (induction) C...T
Group 2	
Galectin 1 (induction)	Sp1/Sp3 binding site
Gai2 (induction)	Sp1/Sp3 binding site
IGF binding protein 3 (induction)	Sp1/Sp3 binding site
p21 ^{Waf1/Cip1} (Cdk2 inhibitor) (induction)	Sp1/Sp3 binding site

FIGURE LEGENDS

FIGURE 1 A. Sites of post-synthetic modifications on the core histones. The structures of the core histones H2A, H2B, H3, and H4 and the sites of modification are shown. The modifications shown are acetylation (Ac), phosphorylation (P), ubiquitination (Ub), and methylation (Me). The enzymes catalyzing reversible acetylation and phosphorylation are shown (HAT, histone acetyltransferase; HDAC, histone deacetylase). B. Crystal structure of the nucleosome (adapted with permission from Dr. Timothy Richmond) (15). C. Chromatin fibers bearing unmodified tails interact, while these interactions are disfavored when the tails are modified.

FIGURE 2 Dynamic histone acetylation is catalyzed by HATs and HDACs.

FIGURE 3 Effect of sodium butyrate on dynamic histone acetylation in MDA-MB-231 human breast cancer cells and avian immature erythrocytes. MDA MB 231 cells were pulse-labeled with [³H] acetate for 15 min and then chased for 0 to 240 min in the presence of 10 mM sodium butyrate. The histones were resolved by AUT PAGE (60 µg of protein in each lane). The left and right panels show the Coomassie Blue stained gel and accompanying fluorogram. The two lanes on the far right contained histones from avian immature erythrocyte histones pulse-labeled with [³H] acetate for 15 min and chased for 60 min in the presence of 10 mM sodium butyrate. S and F are the stained gel and accompanying fluorogram, respectively. The acetylated species of H4 are denoted numerically 0, 1, 2, 3, and 4, representing the un-, mono-, di-, tri-, tetraacetylated species, respectively (adapted with permission (3)).

FIGURE 4 Rates of histone acetylation in MCF-7 (T5) human breast cancer cells cultured under estrogen replete conditions. MCF-7 (T5) cells were pulse-labeled with [³H] acetate for 15 min and then chased for 0 to 240 min in the presence of 10 mM sodium butyrate. The histones were resolved by AUT PAGE (60 µg of protein in each lane). The proportions of total radiolabeled H4, H2B and H3 associated with monoacetylated form were determined by scanning the fluorograms. The proportion of labeled monoacetylated isoforms (H4-Ac1, H3.2-Ac1 and H2B-Ac1) present in total labeled H4, H3 and H2B at zero time was arbitrarily set at 100. The rapid rate of acetylation was determined using the data obtained from the 0 to 20 min butyrate chase period, while the slower rate of acetylation was determined using data from the 60 to 240 min butyrate chase period (adapted with permission (3)).

FIGURE 5 Schematic representation of HDLP interactions with trichostatin A in the crystal structure of a HDAC homologue from the hyperthermophilic bacterium *Aquifex aeolicus*. TSA is in black and the protein is in red. HDLP residues are labeled in red with their counterparts in HDAC1 indicated in black (adapted with permission from Dr. Nikola P. Pavletich (43)). The structures of trichostatin A and butyrate are shown.

FIGURE 6 HDAC multiprotein complexes are recruited to specific genomic sites by regulatory proteins. HDAC1 and HDAC2, together with RbAp46/48, are components of two large multiprotein complexes (Sin3 and NuRD) containing mSin3A/B or CHD3/CHD4, respectively (25).

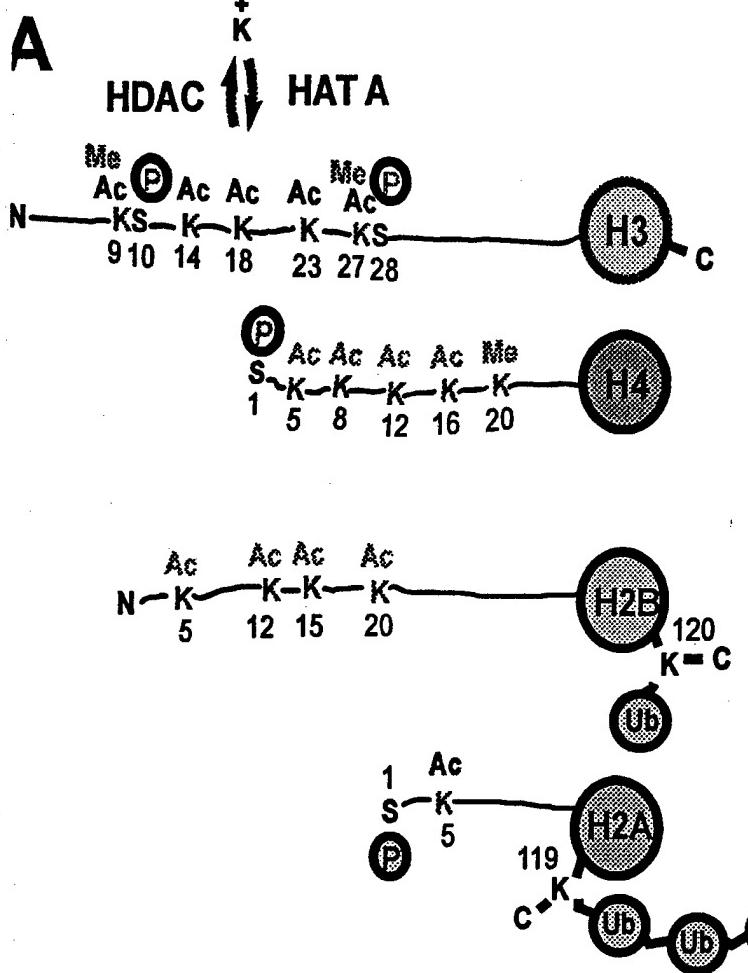
FIGURE 7 Sp1 is not associated with Sp3. MCF-7 (T5) cell lysate was incubated with anti-Sp1 antibodies, and the immunoprecipitation (IP, lane 2) and immunodepletion (ID, lane 3) fractions were collected. The immunodepleted fraction was next incubated with anti-Sp3 antibodies, yielding IP (lane 4) and ID (lane 5) fractions. Proteins of the cell lysate (lane 1), IP and ID fractions were loaded onto a SDS 10% polyacrylamide gel, transferred to nitrocellulose membranes, and immunochemically stained with anti-Sp1 and anti-Sp3 antibodies. The long (L) and short (M1 and M2) forms of Sp3 are identified (adapted with permission (7)).

FIGURE 8 Sp1 and Sp3 are associated with HDAC1 and HDAC2. MCF-7 (T5) cell lysate, IP and ID fractions prepared as described in Fig. 7 were loaded onto a SDS 10% polyacrylamide gel, transferred to nitrocellulose membranes, and immunochemically stained with anti-HDAC1 and HDAC2 antibodies. The arrow points to the HDAC2 species with reduced mobility (adapted with permission (7)).

FIGURE 9 HDAC2 is associated with and phosphorylated by protein kinase CK2. The C-terminal amino acid sequences of mammalian HDAC1 and HDAC2 and the sites of protein kinase CK2 phosphorylation are shown. Equal amounts of MCF-7 (T5) cell lysate was immunoprecipitated with anti-HDAC1, anti-HDAC2 or anti-HDAC3 antibodies. The immunoprecipitated samples and nuclear extracted protein (10 µg) were loaded onto a SDS 10% polyacrylamide gel, transferred to a nitrocellulose membrane and immunochemically stained with anti-CK2α, anti-HDAC1, anti-MBD3 and anti-Sin3 antibodies (adapted with permission (7)).

FIGURE 10 Model for the butyrate induction of the Cdk2 inhibitor, p21^{Waf1,Cip1}. In the absence of butyrate, ZBP-89 and Sp3/Sp1 recruits the p300 and HDAC1,2/CK2 complex, respectively. The steady state level of histone acetylation is low and not supportive of transcription (left panel). When butyrate is present, histone deacetylase activity is inhibited, allowing the histones to become hyperacetylated. The modified chromatin now supports transcription (right panel).

FIGURE 11 The diagram shows the induction of p21^{Waf1,Cip1} gene expression by transient activation of ERK activity. The elevated levels of p21^{Waf1,Cip1} inhibit cyclin E-Cdk2 activity and promote the assembly of stable cyclin D1-Cdk4/6 kinase complexes. The p21^{Waf1,Cip1} gene is repressed and p21^{Waf1,Cip1} protein levels decline, allowing activation of cyclin E-Cdk2. Both cyclin D1-Cdk4,6 and cyclin E-Cdk2 are involved in the phosphorylation of Rb and the release of E2F, which activates the promoters of genes involved in progression through S phase of the cell cycle. Butyrate would induce expression of p21^{Waf1,Cip1}, inhibiting cyclin E-Cdk2 activity and arresting cell cycle progression. ECM, extracellular matrix; RTK, receptor tyrosine kinase.



B Histone Octamer

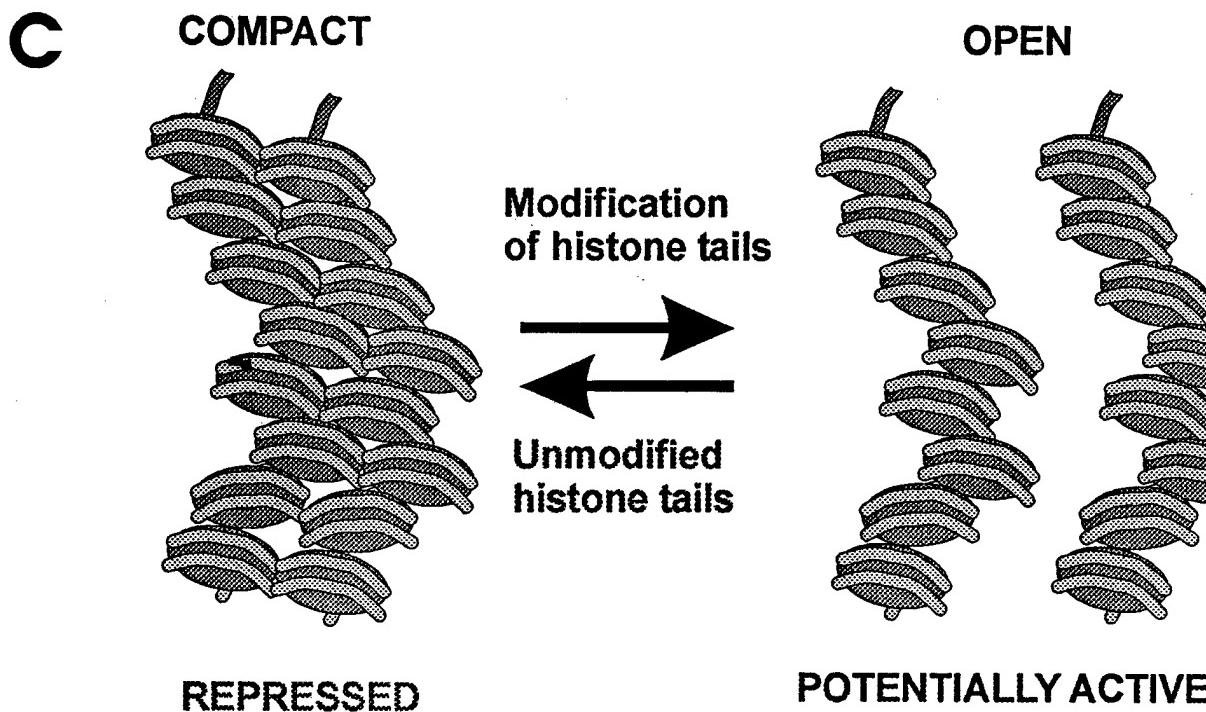
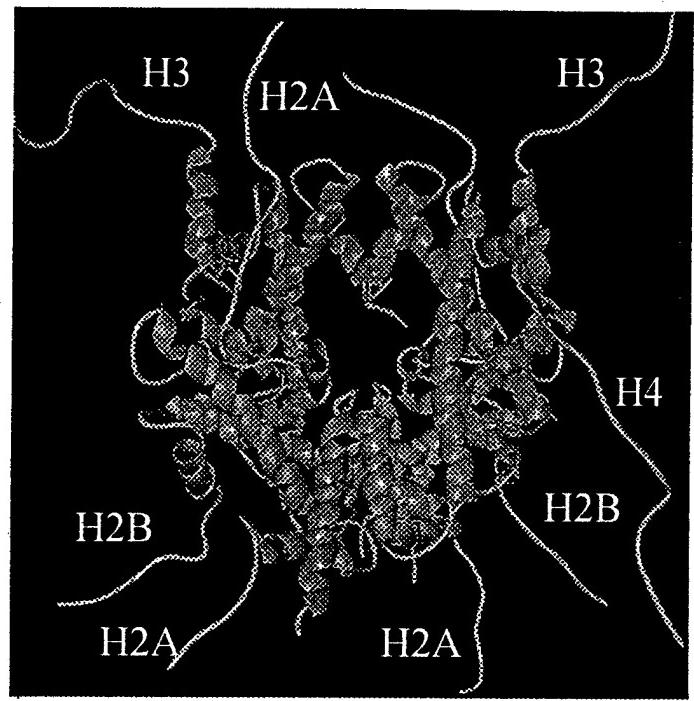


Figure 1

- Potent HATs • Weak HATs
- CBP - SRC1
- p300 - SRC3
- PCAF
- Tip60

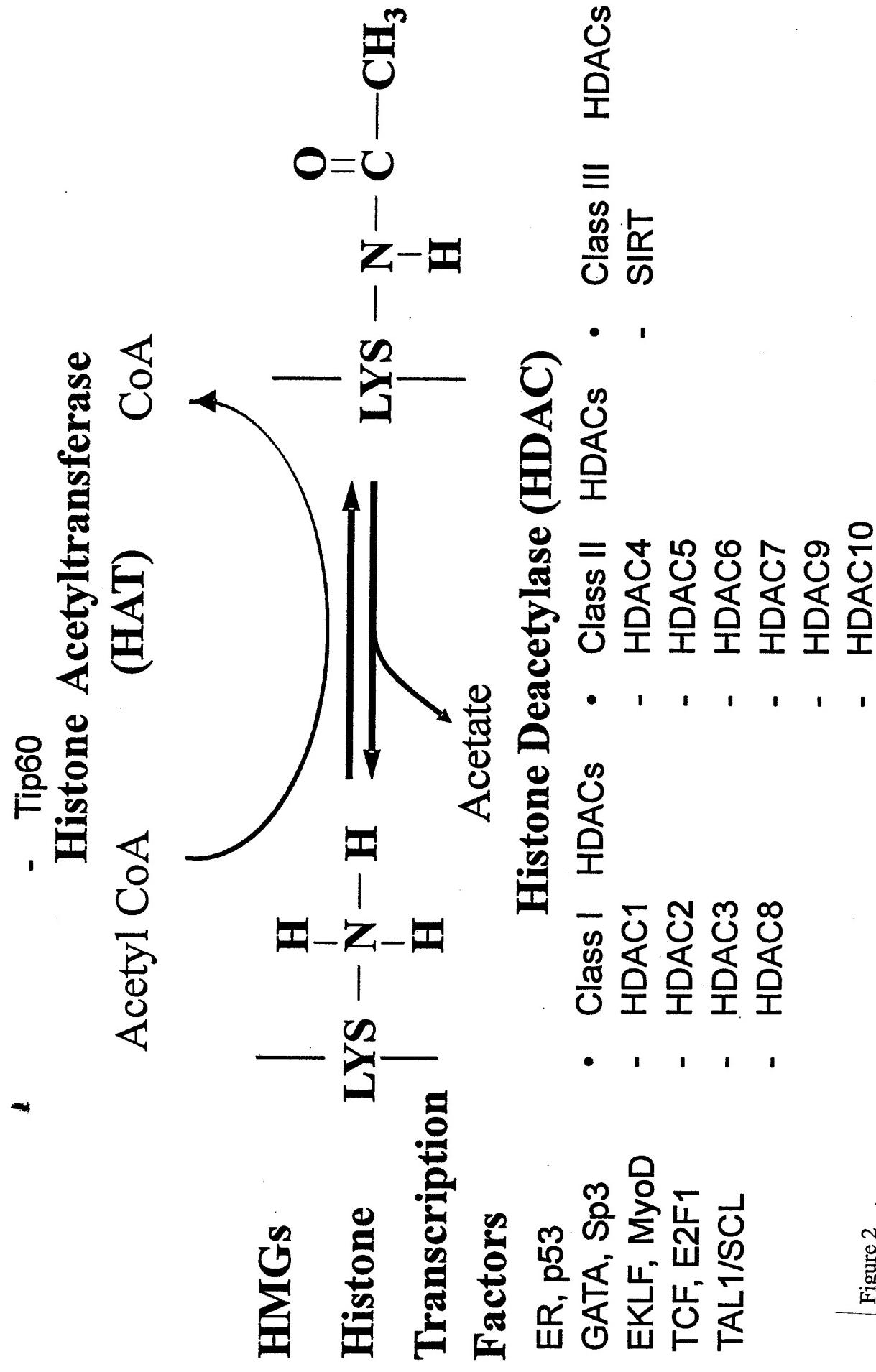


Figure 2

Butyrate and Histone Acetylation Dynamics

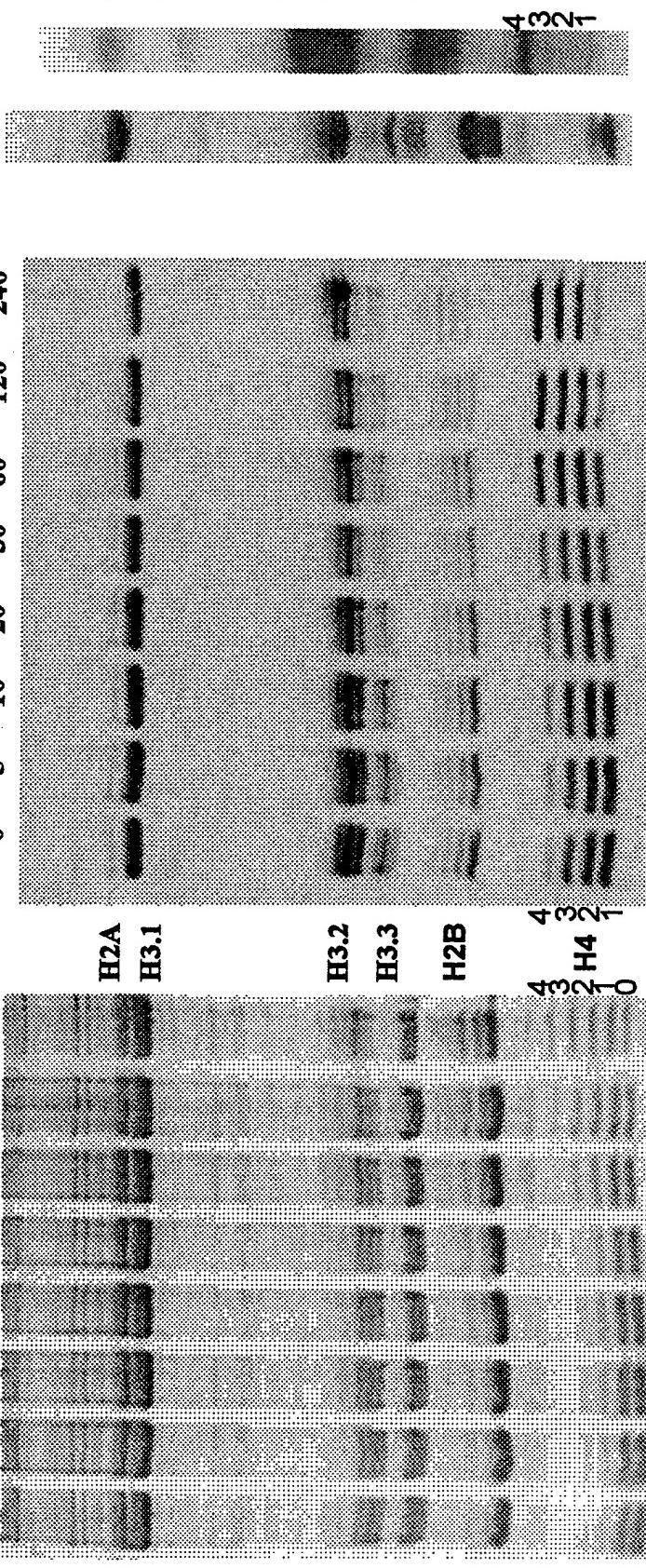
**Histone Acetylation in MDA MB 231 cells
(pulse 15 min)**

Time of chase (min) 0 5 10 20 30 60 120 240

Time of pulse, 15 min pulse,
Avian Erythrocyte

Time of chase (min) 0 5 10 20 30 60 120 240

15 min pulse,
60 min chase



Stain

Fluorogram

S F

Figure 3

Analysis of the rate of histone acetylation in MCF-7 (T5) cells

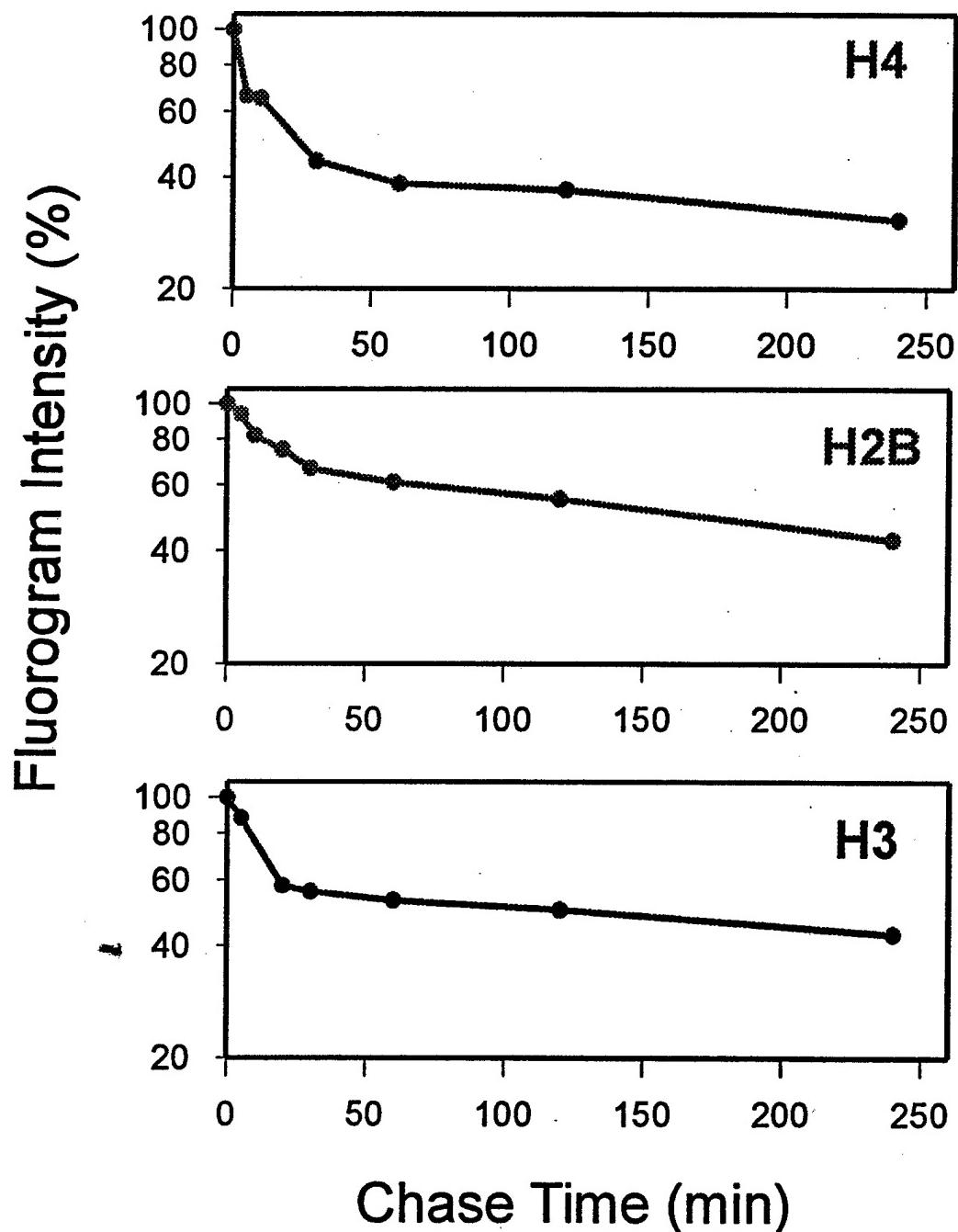
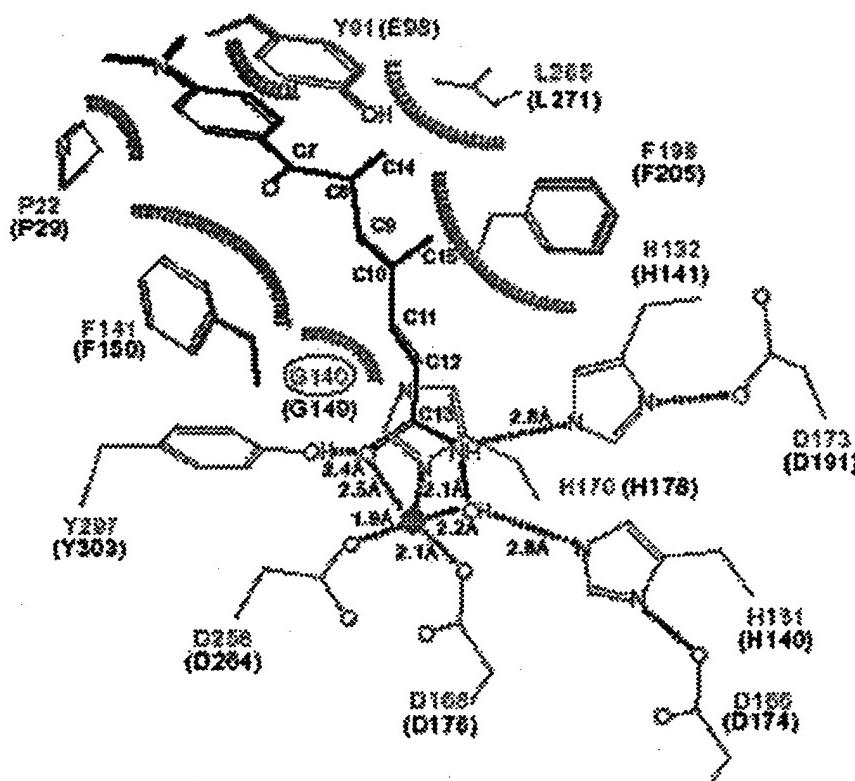


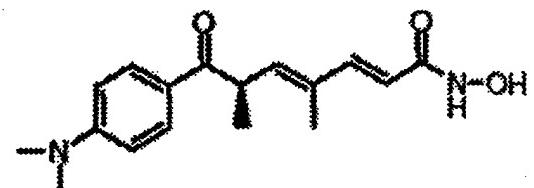
Figure 4

Butyrate – mode of action

hyperthermophilic bacterium *Aquifex aeolicus* HDLP
with trichostatin A



Butyrate



Trichostatin A

Figure 5

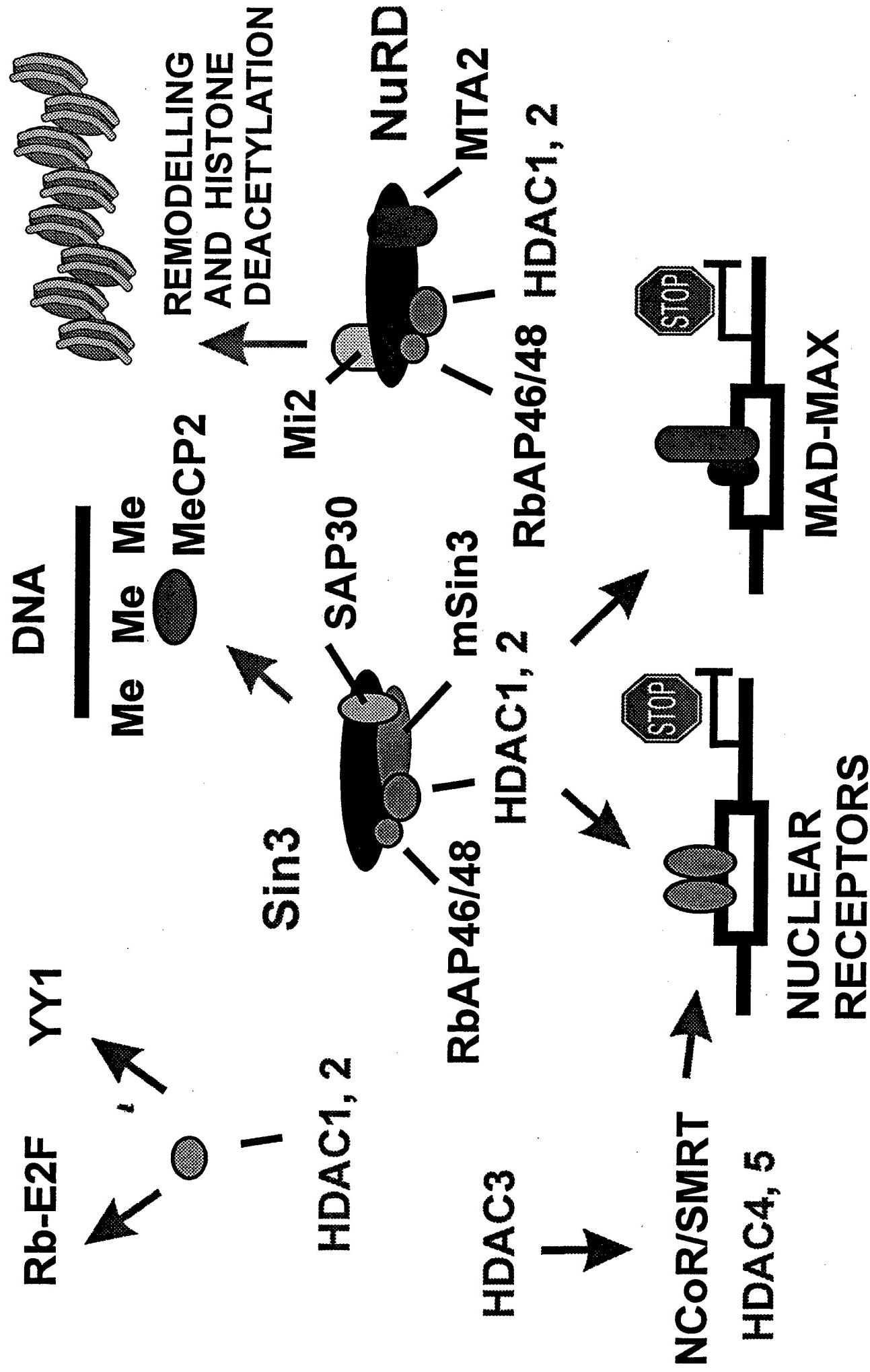
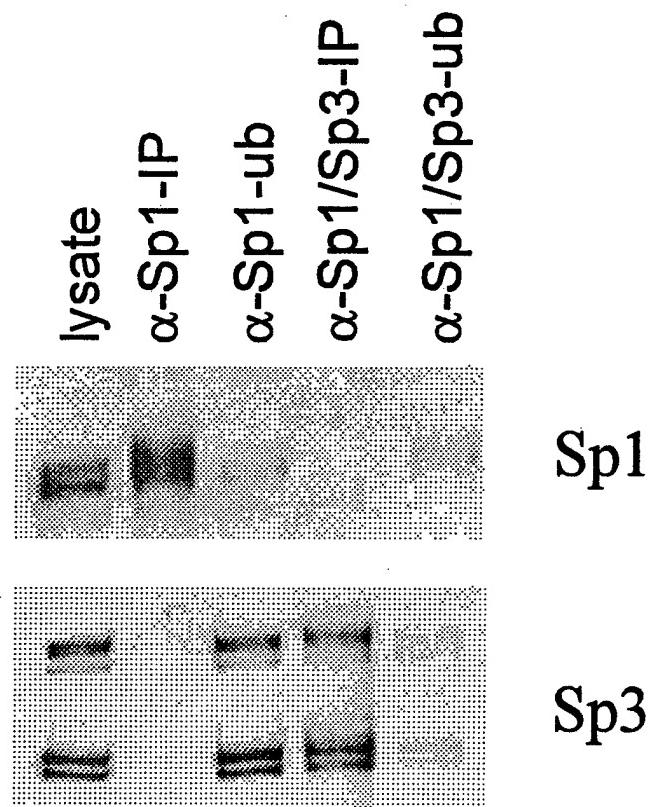
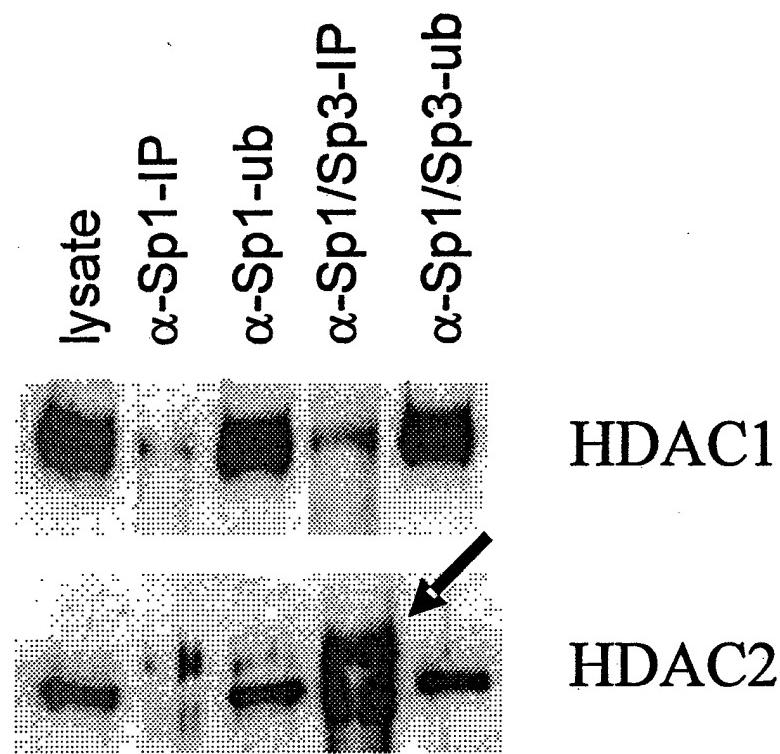


Figure 6



Sp1 does not form complexes with Sp3

Figure 7



**Sp1 and Sp3 form complexes
with HDAC1 and 2, but not HDAC3**

Figure 8

HDAC2 is associated with and phosphorylated by CK2

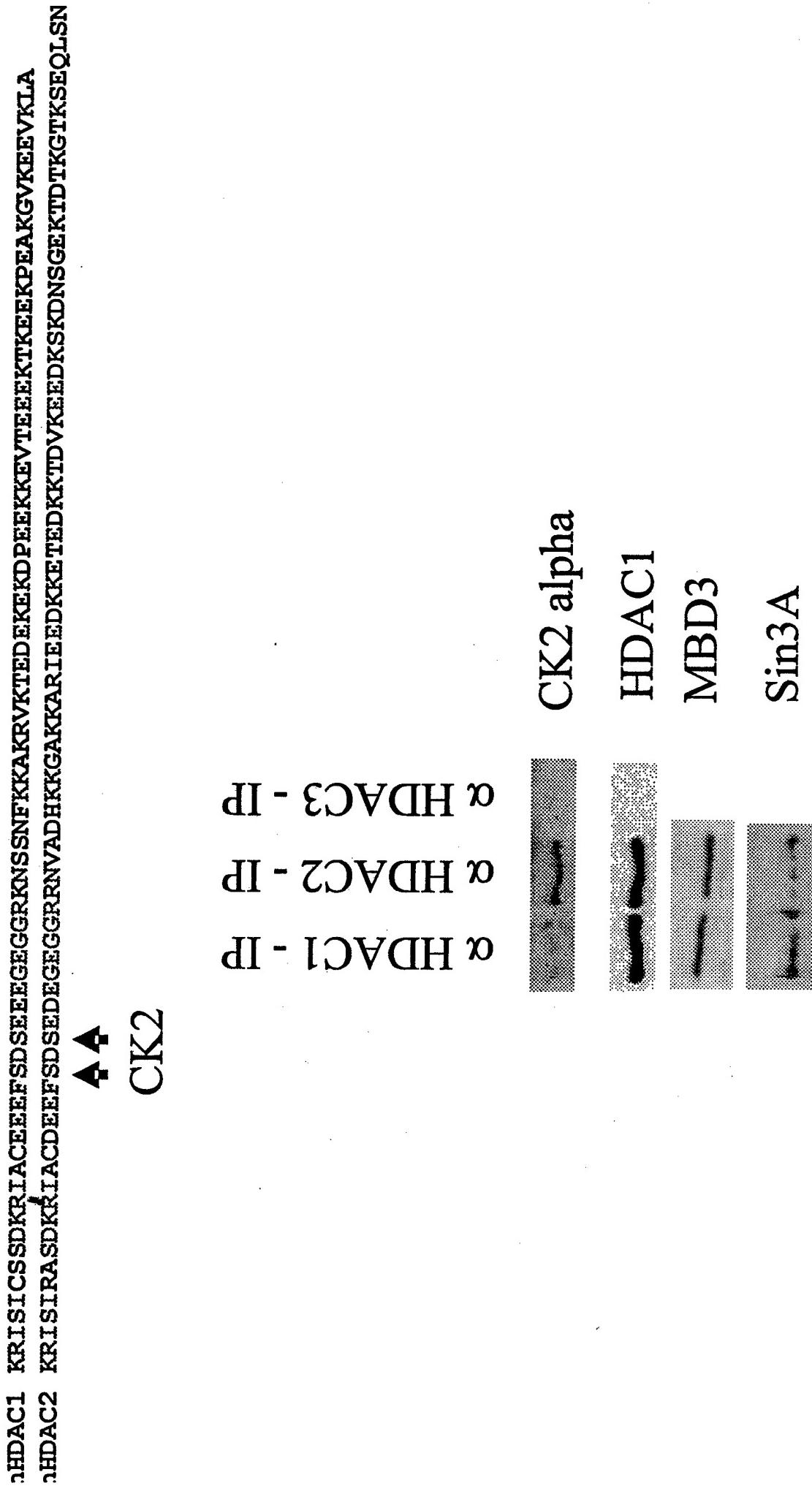


Figure 9

p21 Waf1/Cip1 promoter

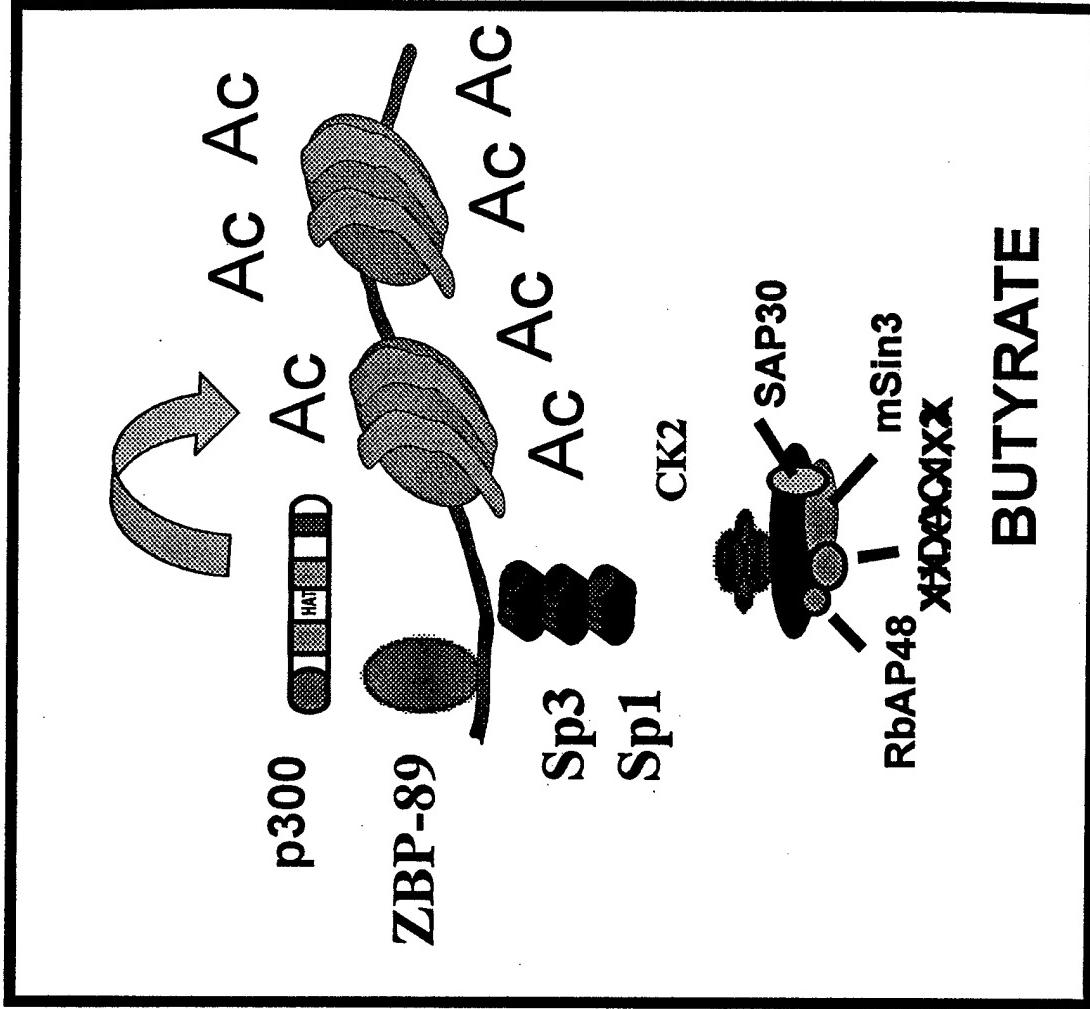
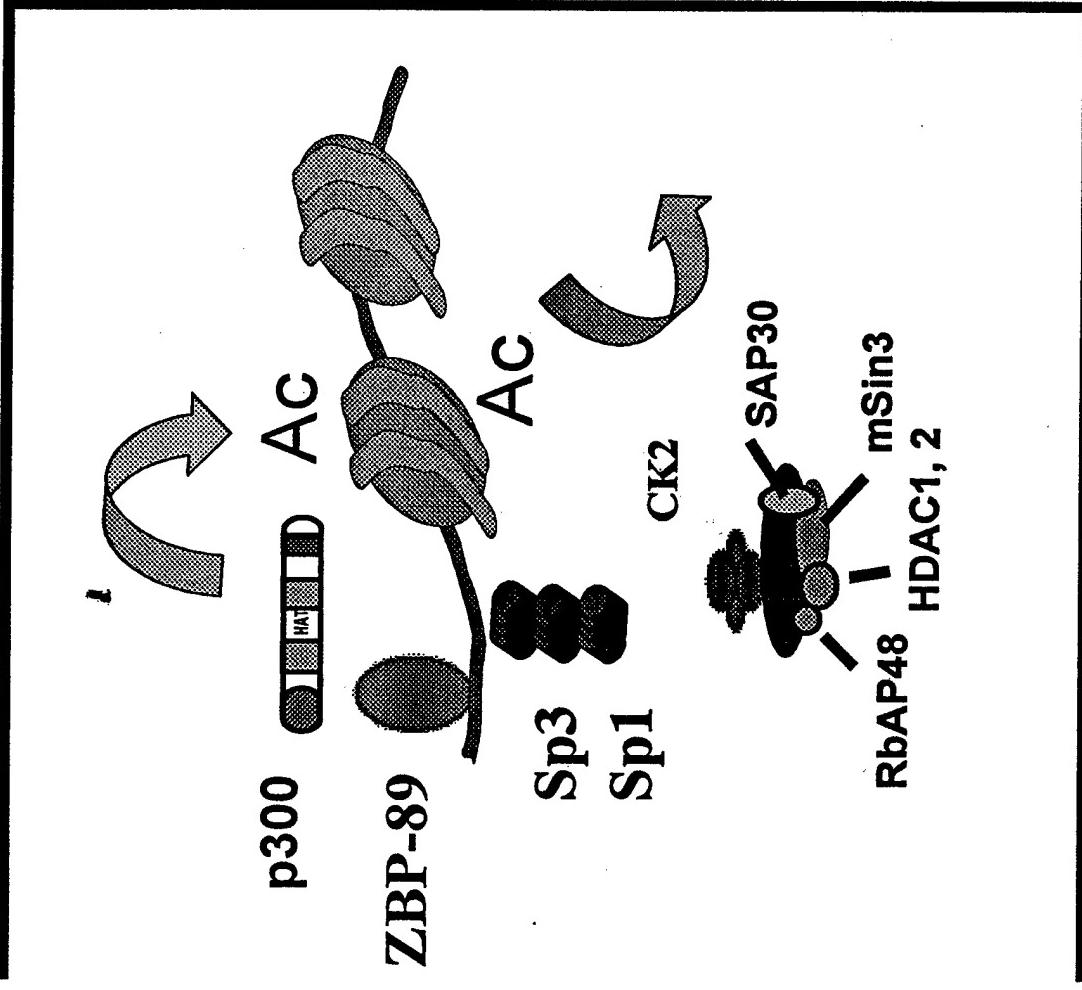


Figure 10

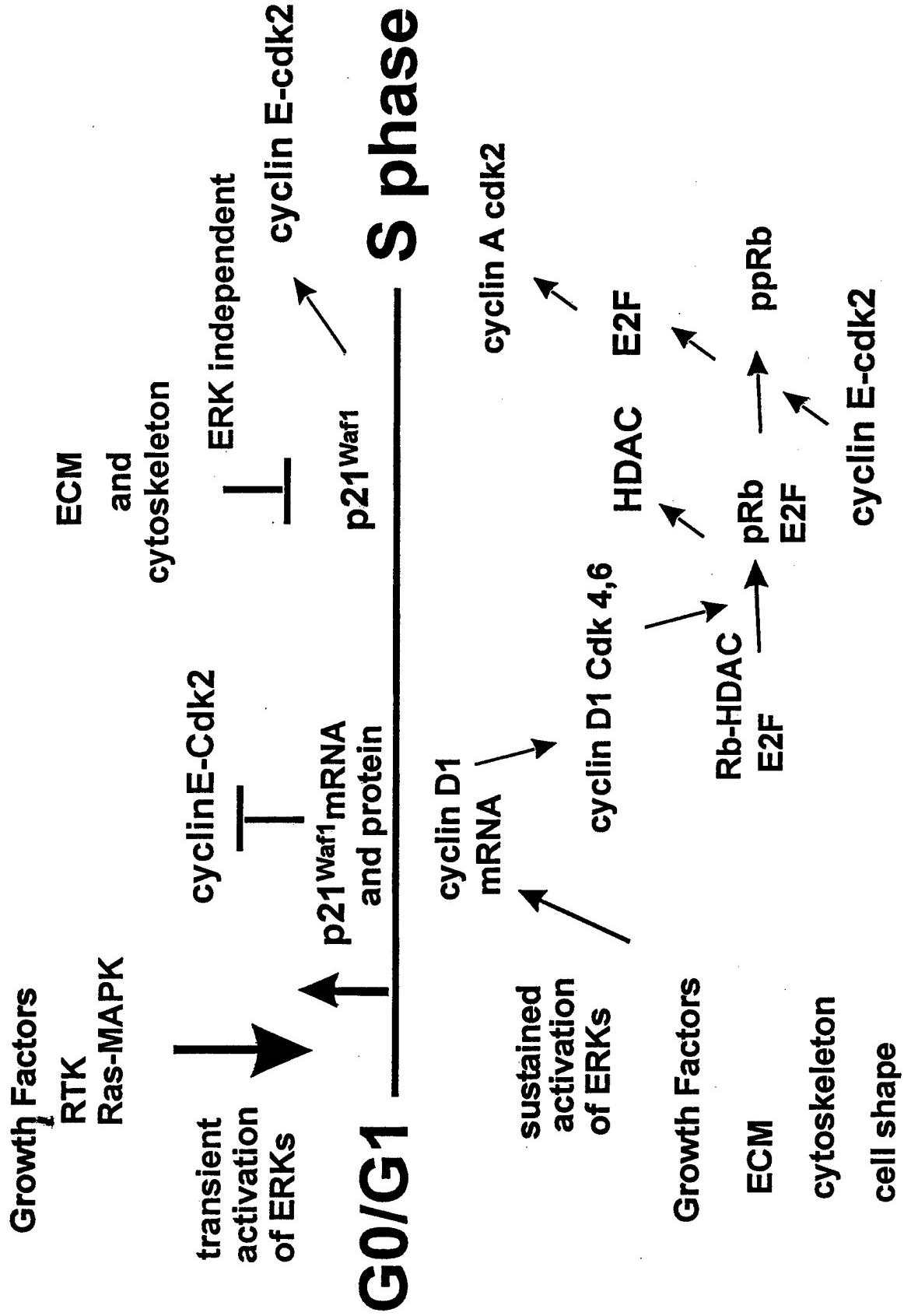


Figure 11